Osteoinduction of human adipose stem cells in 3D hydrogel culture using mechanical stimulation

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TIIVIVTELMÄ

Matala-amplitudisella, korkeataajuisella (LMHF) vibraatiolla on kliinisissä kokeissa ja kaksiulotteisessa (2D) ympäristössä suoritetuilla solukokeilla osoitettu olevan luumuodostusta lisäävä vaikutus. Kolmiulotteiset (3D) soluviljelmät muistuttavat solujen normaalia kasvuympäristöä 2D olosuhteita paremmin ja ovat näin ollen kiinnostava lähestymistapa tutkittaessa vibraation vaikutusta solujen jakautumiseen ja erilaistumiseen. Korkean vesipitoisuuden omaavat, luonnolliset tai synteettiset hydrogeelimateriaalit pystyvät jäljittelemään solun ulkoisia tukirakenteita tarjoten soluille ympäristön toteuttaa niiden fysiologisia tehtäviään. Tärinän aikaansaamien ulkoisten ärsykkeiden voidaan ajatella johtuvan hydrogeeleissä soluille ja aktivoivan luun muodostusta lisääviä signalointireittejä. Tämän työn tarkoituksena oli selvittää 3D hydrogeelien kykyä tukea ihmisen rasvan kantasolujen (hASC) luuerilaistamista korkea-amplitudisen, korkeataajuisen vibraation (HMHF) alaisuudessa.

Soluviljelyolosuhteina toimivat: staattinen kasvatus perus- ja luuerilaistamisliuoksessa sekä vibraatiokasvatus luuerilaistamisliuoksessa. Tutkimuksessa käytetyt rasvan kantasolut olivat peräisin kolmelta eri luovuttajalta. Soluviljelyssä käytettyjen kuoppalevyjen vertikaalinen stimulaatio aikaansaatiin Tampereen teknillisessä yliopistossa suunnitellulla ja valmistetulla laitteella, jonka maksimi kiihtyvyydeksi säädettiin 2,5 g ja taajuudeksi 100 Hz. Vibraatiota toistettiin viisi kertaa vuorokaudessa 150 minuutin välein puolen tunnin jaksoissa. Vibraation ja biomateriaalin vaikutuksia solujen elinvoimaisuuteen analysoitiin elävät ja kuolleet solut erottelevalla värjäyksellä 6 - 7 ja 12 - 13 päivän viljelyn jälkeen. Solujen erilaistumista arvioitiin alkalisen fosfataasin (ALP) aktiivisuuden ja mineralisaation määrityksellä, sekä analysoimalla adipo- ja osteogeenisten geenien ilmentymistä näytteissä.

Rasvan kantasolut säilyivät elinvoimaisina tutkitussa biomateriaalissa, kunnes hydrogeelit alkoivat kutistua. Vibraatiolla ei ollut vaikutusta solujen elinkykyyn, mutta se lisäsi solujen jakaantumista minkä seurauksena geelien kutistuminen lisääntyi. Mekaanisesti stimuloidut solut eivät osoittaneet kohonnutta aktiivisuutta geenien Runx2A, Collagen I ja ALP osalta, sen sijaan adipogeeninen markkeri aP2 ja osteogeeninen markkeri Dlx5 olivat koholla verrattuna staattisesti kasvatettuihin kontrolliviljelmiin. Mineralisaatiota tapahtui kaikissa tutkituissa vibraatiokasvatetuissa viljelmissä, mutta solujen mineralisaatiossa kontrolliviljelmissä oli eroja solulinjojen välillä.

Tulokset osoittivat, että vibraation käytöllä solujen kasvatuksessa voi olla positiivinen vaikutus kollageeni I hydrogeeleissä kasvatettujen hASC:n luuerilaistumiseen, koska Dlx5 geeniä pidetään vahvana osteogeenisenä markkerina. Kuitenkin adipogeenisen aP2 markkerin samanaikaisesti kohonnut geenieksprессio voi olla osoitus vibraatiostimulaation epäspesifisyydestä solujen erilaistumiseen. Lisäksi 3D olosuhteet saattoivat hankaloittaa stimulaation etenemistä soluille. Koesarjojen välillä nähty vaihteleva vaste stimulaatiolle on osittain selitettävissä solulinjakohtaisella vaihtelulla ja osittain kollageeni I geelien kutistumisella.

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ABSTRACT

Low-magnitude high frequency vibration has an ability to stimulate bone formation *in vivo* and *in vitro* 2D cultured stem cells. Highly absorbent hydrogels made from natural or synthetic polymers have been shown to mimic the structure of extracellular matrix offering cells a niche to undertake their physiological functions. The use of the 3D extracellular matrix analogous biomaterials is an attractive strategy for studying the effects of the mechanical stimulation to the osteogenic differentiation of stem cells. External forces caused by vibration are thought to conduct via hydrogel to the cells and activate osteogenic signaling pathways inside the cell. In this study, the capability of Collagen I hydrogel purified from rat tail to host human adipose stem cells (hASC) cultured in three dimension and induce their osteogenic differentiation in osteogenic conditions under the vibration loading were demonstrated. Both, the role of the culture medium and vibration loading on hASC osteogenic differentiation were evaluated.

Cells were cultured in three distinct conditions: in static cultures with the basic medium, static cultures with the osteogenic medium and under the vibration loading in osteogenic medium. hASC used were derived from three different donors. A vertical vibration for cell plates was achieved with high magnitude high frequency vibration loading device, a peak acceleration of 2.5 g, using a sine wave at 100 Hz with an effective vibration period of 30 minutes with 150 min break. Device carried out five vibration periods in 24 hour. Effects of the vibration and biomaterial on hASC viability were evaluated by live/dead staining after 6 - 7 and 12 - 13 days of culture. Also the alkaline phosphatase activity (ALP) and mineralization was measured to assess the osteogenic differentiation of the cells. Furthermore adipo- and osteogenic differentiation of the cells was estimated with real time PCR.

Assays showed sustained cell viability and proliferation throughout the hydrogels over 12 days and moreover. Mineralization occurred by the cells under osteogenic conditions in vibrated cultures. However, there was a difference in mineralization between separate cell lines in the static control cultures. Exposure to the vibration seemed to speed up the proliferation of hASCs. However, mechanically stimulated cells did not show increased expression of key bone forming genes Runx2, Collagen I and ALP as quantified by RT-PCR. Instead, expression of adipogenic marker gene aP2 and osteogenic marker gene Dlx5 were increased in vibrated cells compared to static cultures.

These findings imply that vibration loading may enhance osteoblast differentiation in hASCs seeded in collagen I hydrogel, since Dlx5 is a strong marker of osteogenesis. However, simultaneous induction of adipogenic gene expression may indicate that the vibration stimulation is not very specific stimulant of differentiation or that the 3D setting hinders the stimulus. Variable cellular responses elicited could be partly caused by donor variation and partly by collagen I hydrogel shrinkage due to the increased proliferation and pulling forces created by the growing cell population.

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Eveliina Koivuniemi,
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TABLE OF CONTENTS

ABBREVIATIONS

ALP	alkaline phosphatase
AM	acetoxymethyl
aP2	human adipocyte fatty acid-binding protein 2
ASC	adipose stem cell
BM	basal medium
BMSC	bone marrow mesenchymal stem cell
bov-COL I	collagen type I from bovine
BSA	bovine serum albumin
CEBP	C/CAAT enhancer binding proteins
COLL1	collagen type Iα1
CREB	cyclic AMP response element-binding protein
DAPI	4'6-diamidino-2-phenylindole
DLX5	distal-less homeobox 5
DMEM	Dulbecco's Modified Eagle Medium
ECM	extra cellular matrix
ESC	embryonic stem cells
EthD-1	ethidium homodimer-1
FA	focal adhesion
FABP4	fatty acid binding protein 4
GLUT4	glucose transporter 4
HA	hydroxyapatite
hASC	human adipose stem cell
HA - HA	Hyaluronan-based hydrazone crosslinked hydrogel
HACDH	carbonyldihydrazide modified hyaluronic acid
HALD 1	aldehyde modified hyaluronic acid
hBMSC	human bone marrow mesenchymal stem cell
HFSC	human fat stem cells
HMHF	high magnitude high frequency
hMSC	human mesenchymal stem cell
HS	human serum
HSC	hematopoietic stem cells
iPSC	induced pluripotent stem cell
LMHF	low magnitude high frequency
LPL	lipoprotein lipase
MSC	mesenchymal stem cell
OM	osteogenic medium
PBS	phosphate buffered saline
PFA	paraformaldehyde
PPARγ	peroxisome proliferator-activated receptor gamma
qALP	quantitative alkaline phosphatase assay
qRT-PCR	quantitative real time polymerase chain reaction
rat-COL I	collagen type I from rat tail
Runx2	Runt-related transcription factor 2
SVF	stromal vascular fraction
TE	tissue engineering

1 INTRODUCTION

The regenerative capacity of the bone tissue is not enough to heal the large bone defects caused by tumor resection, trauma or infection. Multiple bone tissue diseases, e.g. osteoporosis, osteogenesis imperfecta or osteomalasia also cause conditions where bone cannot heal itself effectively (Lewiecki 2000). Bone tissue engineering (TE) is an approach that combines cells, biomaterials and osteoinductive, bone forming factors to produce new bone to substitute deceased or traumatically lost bone tissue.

Mechanical signals play an important role in the skeleton as the musculoskeletal tissue responses to the changes by maintaining the structure or by increasing tissue growth (Qin and Hu 2014). Absence of mechanical stimulus like functional loading results in the loss of bone metabolism and bone mass (Robling and Turner 2009, Young et al. 2013). Mechanotransduction is a process where the cell converts mechanical signals into biochemical signals. Sensed signals initiate signal cascades inside the cell and are transferred between the cells. Various cells of the musculoskeletal system, including osteocytes, osteoblasts and mesenchymal stem cells (MSCs), have been shown to be responsive to mechanical stimulus (Robling and Turner 2009).

Human adipose stem cells (hASCs) have proven to be good choice for the bone tissue engineering applications owing to their many pros versus other cell sources (Lindroos et al. 2011). Previously, it was also shown in a study conducted in University of Tampere that human adipose stem cells are sensitive to mechanical stimulus such as vibration loading under osteogenic differentiation (Tirkkonen et al. 2011). However, so far the effects of mechanical stimulus to osteogenic differentiation of MSCs have mostly been studied in two-dimensional (2D) cultures (Kim et al. 2012, Tirkkonen et al. 2011).

Hydrogels have emerged as interesting materials for cell culture and bone tissue engineering due to their desirable physical characteristics and unique biocompatibility (Amini et al. 2012). These types of biomaterials do not only serve as matrices for TE but also are capable of mimicking extracellular matrix topography in three-dimensional (3D) cultures and can easily transmit mechanical stimulus such as vibration to the cells (Amini et al. 2012, Huang et al. 2012). Collagen I is one of the most abundant fibrillary molecule in the bone tissue extracellular matrix (ECM) and can be easily extracted from bovine and porcine skin and from rat tail joint thus providing good hydrogel source for hASC's osteogenesis (Egeblad et al. 2010, Kreger et al. 2010).

This master thesis work studied the effects of mechanical loading on adipose stem cells cultured in 3D matrix and their differentiation into osteogenic cells. Stem cells were seeded in 3D hydrogels and the morphology and viability of hASCs cultured in three different hydrogels was compared. Hydrogel with the best properties was selected into the mechanical vibration loading experiment. In vibration loading the cell - hydrogel cultures were exposed to high frequency high magnitude (HMHF) vibration using a loading device designed and built previously at Tampere University of Technology (TUT).

This study was based on the hypothesis that vibration loading enhances osteogenic differentiation of hASCs and that stimulation system used in previous study by Tirkkonen et al. can be translated from 2D to 3D environment. This included a hypothesis that the selected hydrogel matrix is able to transmit mechanical signals to the cells.

2 REVIEW OF THE LITERATURE

2.1 Bone tissue engineering

Healthy bone tissue regenerates well, but the sick, frail or infected bone does not repair itself effectively, either large bone defects do not heal spontaneously in the body. Typically, bone defects, as a result of the disease or trauma have been replaced with the autologous bone grafts origin from the patient or with allografts from the human cadaver. Problems with autologous grafts are often poor quality, pain at the donation site and the deficiency consisting of donation area (Jakob et al. 2012). As for allografts the biggest problems are the possible immune reaction or infections caused by the transplant in the receiver (Jakob et al. 2012). Due to the poor regenerative capacity of damaged bone tissues, the shortcomings of the existing therapies and poor availability of replacement tissue, other possibilities to replace the damaged tissue have been explored.

Tissue engineering is a concept introduced four decades ago and it refers to the replacement or repair of the missing or damaged tissue with the functional tissue constructed out of supportive structure, commonly known as a scaffold, cells and biologically active molecules *in vitro*, outside the body or *in vivo*, in the body (Rustad et al. 2010, Vacanti and Langer 1999, Young et al. 2013). Scaffolds made from different biomaterials act as carriers of the cells and the growth factors to the site of damage and control the growth and development of the cells and new tissue. At its best, these 3D structures function as an ECM; protect cells and control cell migration, proliferation and differentiation (Kruger et al. 2013). Structure and the material of the scaffold should enable the access and removal of metabolic products and nutrients, as well as angiogenesis (Kruger et al. 2013). Growth factors can be implanted in the scaffold with the cells or they can be secreted by cells which naturally produce and excrete such molecules (Zanetti et al. 2013, Kruger et al. 2013). The biomaterials used in regenerative medicine are typically classified into natural and synthetic polymers, ceramics, glasses and the composites of the itemized (Zanetti et al. 2013). In addition to the material and design choices of the scaffold other matters taken into consideration when planning to substitute bone tissue are the cell source and the osteogenic differentiating conditions of the cells.

2.2 Stem cells

Stem cells are potential cell source to the TE applications and research due their ability to self-renew and differentiate in multiple cell lines (Choumerianou et al. 2008). Stem cells are typically classified into four groups based on their differentiation capacity: unipotent,

multipotent, pluripotent and totipotent cells. Furthermore the source of the cells also has an effect on stem cell capabilities. Figure 1 presents different sources of stem cells and their differentiation capacity. Totipotent cells are able to differentiate into any cell or tissue of adult organism and form a complete embryo and extraembryonic tissue. In fact, only a one-cell embryo (zygote) and 2-cell stage embryo are totipotent in both senses (Condic 2014). Totipotency is lost by the time cells reach the blastocyst stage after few days from fertilization (Condic 2014). Cells isolated from the inner cell mass of blastocysts are capable to differentiate into all cell types of three embryonic layers developed during gastrulation: ectoderm, endoderm and mesoderm (Choumerianou et al. 2008). These cells are termed as pluripotent. Totipotent and pluripotent cells originate from embryo and are thus also called embryonic stem cells (ESCs). Additionally induced pluripotent stem cells (iPSC) are used in research. These cells are originally adult somatic, not germ line cells reprogrammed via viral transfection to resemble pluripotent ESCs (Takahashi and Yamanaka 2006). In the adult individual, almost any tissue has also been found to harbor stem cells (Choumerianou et al. 2008). These cells are able to differentiate into multiple cell lineages, presumable restricted to the cell types of the tissue origin and thus termed as multipotent (Choumerianou et al. 2008). Also stem cells isolated from the fetal tissue (e.g. Umbilical cord blood (UCB) or amniotic fluid (AF)) have this same fine property as cells purified from the adult tissue (In 't Anker et al. 2004). Unipotent cells are also adult stem cells, but capable of differentiate only towards one cell type. Multipotent stem cells from adult tissue are classified in three categories according to germ layer they originate (Choumerianou et al. 2008). First, cells of endodermal origin: pulmonary epithelial stem cells, gastrointestinal tract stem cells, pancreatic stem cells, hepatic oval cells and mammary and genital gland stem cells. Second, the cells of ectodermal origin: neural stem cells, skin stem cells, and ocular stem cells. Third, the cells of mesodermal origin: bone marrow hematopoietic and stromal stem cells (Choumerianou et al. 2008).

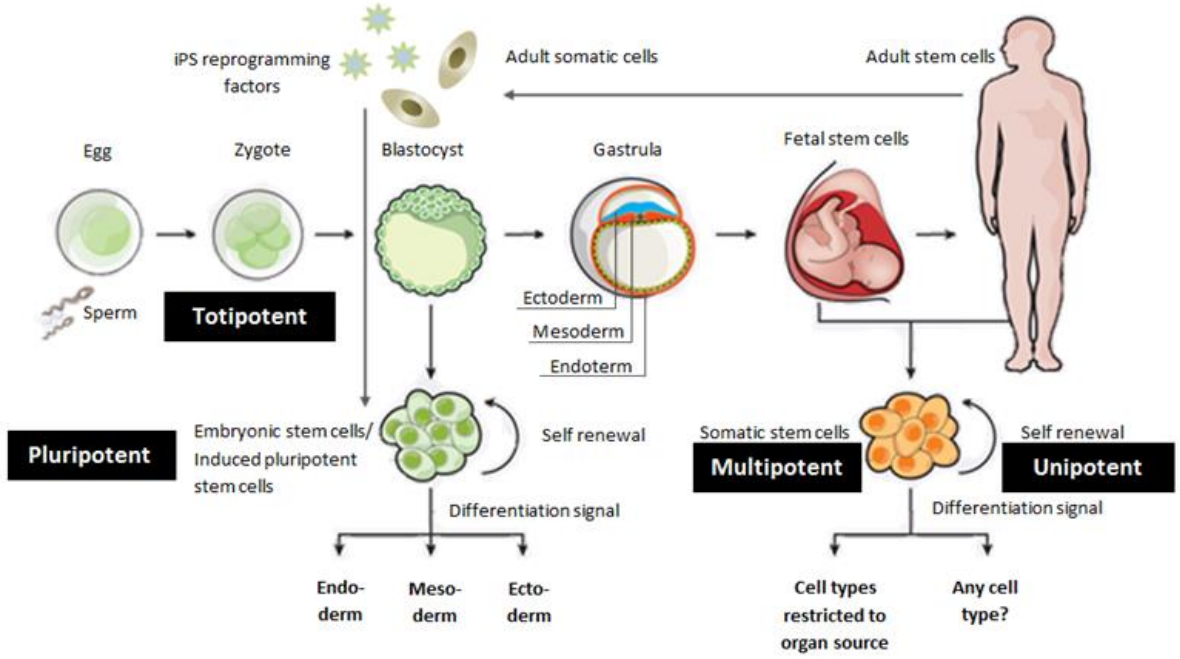


Figure 1 Different sources and potency of stem cells. During gastrulation, the embryo develops three germ layers (endoderm, mesoderm, and ectoderm), which differentiate into distinct tissues. Image modified from (Shevde 2012).

Embryonic stem cells and iPS cells possess better differentiation capacity than adult stem cells, but ethical, legal and political cares as well as safety issues concerning the use of these cells in research favor stem cells from adult tissue (Pera and Hasegawa 2008, de Wert and Mummery 2003). Stem cells from mesodermal germ line e.g. hematopoietic stem cells (HSCs) and mesenchymal stem cells have been under the interest for decades (Choumerianou et al. 2008). In this section the multipotent adult stem cells are presented mainly focusing in the ones origin from adipose tissue.

2.3 Mesenchymal stem cells

The first adult stem cells discovered were HSCs from mesodermal origin. These bone marrow residing cells were described already in early 1960s by Till, McCulloch, and colleagues (1964). Soon after that in (1968), Friedenstein and co-workers discovered non-hematopoietic MSCs also isolated from bone marrow. Plastic adherent, colony forming bone marrow-derived mesenchymal stem cells (BMSC) were rapidly adopted for the research and later on for clinical use. However, the clinical use of BMSCs has presented problems, including painful harvesting procedure and low number of cell upon bone marrow aspirate (Pittenger 2008). This has led many researchers to investigate alternate sources for MSCs. Mesenchymal stem cells with similar characteristic than BMSCs have now been isolated from several organs, such as adipose tissue, dental pulp, umbilical cord and placenta (Avram et al. 2007, da

Silva Meirelles et al. 2006). Although little is known about the *in vivo* signals that orchestrate MSCs tissue maintenance and repair, these cells have become increasingly attractive tools for the study of early differentiation pathways *in vitro,* since MSCs derived from a variety of sources easily proliferate under experimental conditions, retain their multipotency, and display low variability between different donors (Avram et al. 2007, Pittenger 2008). Multiple studies conducted have also revealed other commonalities like support of the hematopoiesis, immunomodulatory properties and secretion of trophic factors between MSCs from different sources (Murray et al. 2014). However, these cells also possess some variations in surface marker expression, immunomodulatory properties and differentiation potential (Murray et al. 2014).

Because of the variable nature and origin and thus heterogenic population of MSCs the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) determines minimal criteria to define human MSC (Dominici et al. 2006). First, MSC must be plastic-adherent when maintained in standard culture conditions. Second, MSCs must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules. Third, MSC must differentiate to osteoblasts, adipocytes and chondroblasts *in vitro*. Furthermore, debate about the true stem cell potential of MSCs from different sources has led to the ISCT to propose the use of term multipotent mesenchymal stromal cells instead of mesenchymal stem cells (Dominici et al. 2006). Both terms appear in literature and are abbreviated as MSCs.

2.4 Adipose stem cells

On the turn of the 21st century the presence of multipotent cells also in adipose tissue was proven (Zuk et al. 2002). Like MSCs, after discovery adipose stem cells were also identified with multiple different names, such as adipose-derived stem/stromal cells (ASCs), adipose derived adult stem/stromal cells (ADASCs/ADSCs), adipose tissue derived mesenchymal stem cells (AT-MSCs) and many others, until the International Federation of Adipose Therapeutics and Science (IFATS) agreed on the use of the acronym ACS to unify nomenclature (Daher et al. 2008). ASCs are considered as MSCs since they fulfil the criteria defined by ISCT discussed in chapter above (Dominici et al. 2006). In addition ISCT and IFATS have also created a joint statement for the characterization of ASCs: In culture, ASCs express markers in common with other mesenchymal stem cells, including CD90, CD73, CD105, and CD44 and remain negative for CD45 and CD31 and they can be distinguished

from bone-marrow-derived MSCs by their positivity for CD36 and negativity for CD106 (Bourin et al. 2013).

Several studies have compared the proliferation and differentiation capacity of BMSCs and ASCs as well as other features these cells exhibit. Adipose stem cells have demonstrated same differentiation capability in right circumstances than BMSCs, but since their harvesting is easier in large quantities with simple surgical procedure serve adipose stem cells a good, reproducible choice for musculoskeletal regenerative medicine applications (Gimble et al. 2011, Zuk et al. 2002, Lindroos et al. 2011). ASCs have also proven to release immunosuppressive factors and these immunomodulatory features suggest that allogeneic and autologous ASCs will engraft successfully for tissue regeneration purposes (Gimble et al. 2011). Furthermore, the differentiation and expansion potential of ASCs can be modified by use of growth factors, bio-inductive scaffolds, and bioreactors providing environmental control and biophysical stimulation (Gimble et al. 2011). Hence ASC's attractiveness is based on the ease with which they can be isolated from the patient's adipose tissue, expanded many fold *in vitro*, manipulated, and reinfused to the same patient or used as an autologous grafts.

Clinical trials

At the moment 87 clinical trials using adipose-derived cells, conducted in 15 countries have been registered with the National Institutes of Health (NIH), about half of which are Phase I or Phase I/II safety studies (www.clinicaltrials.gov). Eight of these studies are related to bone tissue and approaches mostly using autologous stem cell injections to the impaired area (www.clinicaltrials.gov).

The clinical cases with hASC-based bone tissue engineering treatments are still limited to few case reports. Majority of these reports relate to craniomaxillo-facial bone defects (Lendeckel et al. 2004, Mesimaki et al. 2009, Taylor 2010, Thesleff et al. 2011, Wolff et al. 2013, Sandor et al. 2013, Sandor et al. 2014). Pak (2011, 2012) demonstrated in his studies that hASC combined with platelet rich plasma, hyaluronic acid and calcium chloride could be used in bone regeneration in a weight bearing sites, like treatment for femoral head osteonecrosis and osteoarthritis.

2.4.1 Isolation of adipose stem cells

Adipose tissue is a source of heterogeneous stromal vascular fraction cells (SVF) and more homogeneous adipose-derived stem cells (Lindroos et al. 2011). Adipose stem cells and SVF can be isolated from minced adipose tissue sample by an enzymatic digestion and following centrifugation (Zuk et al. 2002). After that ASCs can be selected from the SVF based on their

plastic adherence (Zuk et al. 2002, Bourin et al. 2013). Adipose stem cells have been isolated from subcutaneous fat harvested both by tissue resection and by liposuction (Barzelay et al. 2015). The yield or characteristic of ASCs do not differ upon the harvesting procedure, but liposuction is less invasive and provides ready minced tissue fragments and therefore may allow more efficient enzymatic digestion (Barzelay et al. 2015).

2.4.2 Adipose stem cell culture

Typically the standard culture medium used for ASCs consist of a commercial base, such as Dulbecco's Modified Eagle Medium/Ham's Nutrient Mixture F-12 (DMEM/F-12), supplemented with 10 % serum (fetal bovine serum, FBS or fetal calf serum, FCS), 1 % antibiotics and 1% L-glutamine (Zuk et al. 2002, Tirkkonen et al. 2011). Instead of using serums from animal origin human serum (HS) is recommended when considering clinical applications (Lindroos et al. 2011, Tirkkonen et al. 2011). Notable is that serum concentrations and origin might have differential effect on the hASC's growth, proliferation, differentiation capacity and gene expression (Witzeneder et al. 2013, Lindroos et al. 2010). Another option for clinical hASC cultures could be a use of serum-free medium and xeno- and serum-free (XF/SF) culture conditions to overcome limitations like availability problems and variability of human serum (Lindroos et al. 2009, Patrikoski et al. 2013).

2.5 Differentiation potential of MSCs

Similarly to other MSCs the multipotent differentiation capacity of hASCs at least towards three lineages is one criteria set by ISCT (Dominici et al. 2006). ASCs can differentiate at least to mesodermal lineages such as adipocytes, osteoblasts and chondrocytes which is enough to verify the multipotency (Zuk et al. 2002). Differentiation of ASCs to adipocytes and osteocytes will be covered in this chapter.

2.5.1 Adipogenic differentiation

Adipogenesis, presented in figure 2 is divided in two main steps: hASC commitment to the preadipocyte fate, followed by the conversion of preadipocytes into mature adipocytes (Avram et al. 2007). *In vitro* adipogenic differentiation of hASCs is achieved under appropriate factors like dexamethasone, insulin, isobutylmethylxanthine (IBMX), biotin, pantothenate and indomethasin (Zuk et al. 2002, Avram et al. 2007). Furthermore the adipogenesis of ASCs can be enhanced with several growth factors including fibroblast growth factor 2 (FGF-2) and insulin-like growth factor 1 (IGF-1) as well as with high plating density and low ECM stiffness (Kakudo et al. 2007, Ghosh et al. 2010, Park et al. 2011).

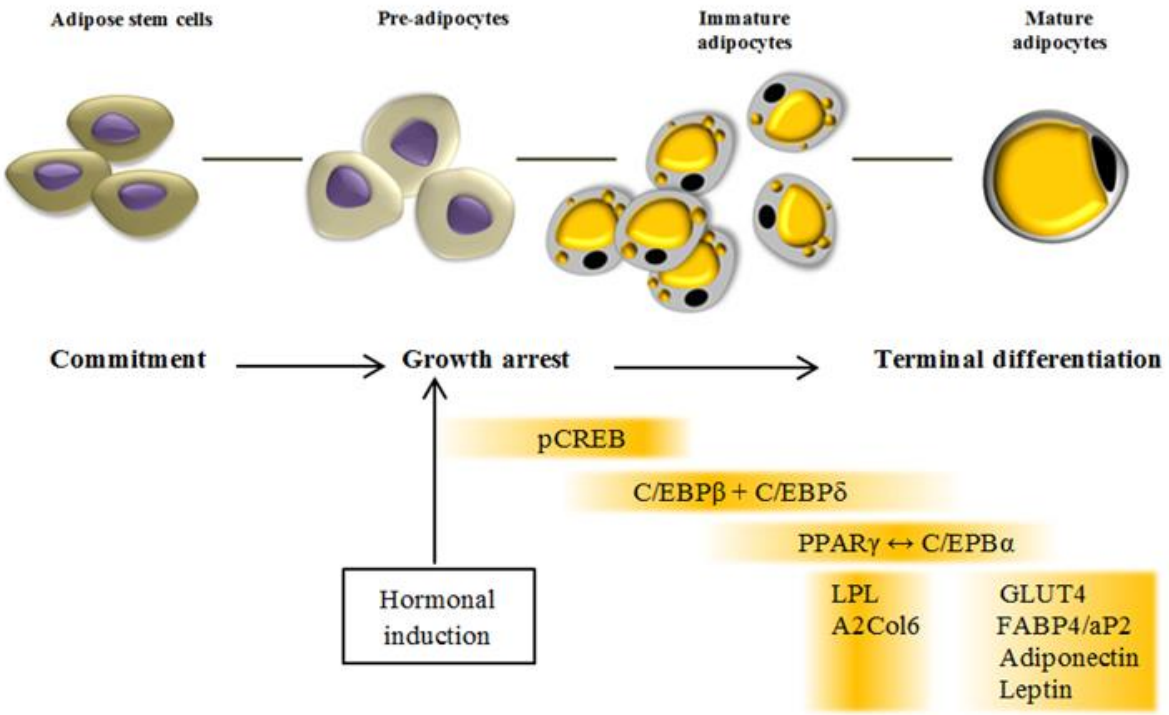


Figure 2 The adipogenic differentiation process and expression pattern of adipogenic markers. Preadipocytes proliferates until they reach confluency and growth arrest. Differentiation phase can be initiated with hormonal inducers.

When differentiated, ASCs accumulate lipid vacuoles which can be detected for example with Oil Red O staining and demonstrate enhanced gene expression of early, mid, and later markers of adipogenesis: cyclic AMP response element-binding protein (CREB), C/CAAT enhancer binding proteins beta and delta (CEBPβ/CEBPδ), peroxisome proliferator-activated receptor gamma (PPARγ), C/CAAT enhancer binding protein alpha (CEBPα), lipoprotein lipase (LPL), adiponectin, leptin, glucose transporter 4 (GLUT4) and fatty acid binding protein 4/ adipocyte protein 2 (FABP4/aP2) (Young et al. 2013, Moseti et al. 2016). PPARγ and CEBPα co-operate to induce adipogenic genes established in mature adipocytes, while PPARγ is considered as main regulator of adipogenesis since CEBPα can't stimulate normal adipogenic maturation in the absence of PPARγ (Moseti et al. 2016, Rosen et al. 1999, Zuo et al. 2006).

2.5.2 Osteogenic differentiation

Differentiation of ASCs towards osteoblast phenotype is a complex process which can proceed via two different routes: by a direct differentiation to osteoblasts (intramembranous ossification) or through a cartilage intermediate step (endochondral ossification) (Long 2011). Currently the majority of the bone TE approaches *in vitro* rely on the direct intramembranous ossification and this route is more closely covered in this overview on osteogenesis. *In vitro*

osteogenesis of ASCs (Figure 3) can be achieved with biochemical stimulation, as medium supplemented with ascorbic acid, β-glycerophophate, dexamethasone and/or 1.25-dihydroxyvitamin D_3 (Zuk et al. 2002, Kyllonen et al. 2013). Osteogenic differentiation of MSCs can also be stimulated by growth factors, such as bone morphogenetic protein 2 (BMP-2) and vascular endothelial growth factor (VEGF) which both have important role in promoting osteoblast differentiation (Hu and Olsen 2016, Long 2011). Furthermore some biomaterials like β-tricalcium phosphate (β-TCP) and material properties as matrix stiffness can promote osteogenic differentiation of MSCs (Reilly and Engler 2010, Duarte Campos et al. 2015, Haimi et al. 2009, Park et al. 2011). Mechanical stimulation, such as stretching, fluid shear stress and vibration loading has also proven to enhance osteogenic differentiation of ASCs (Tirkkonen et al. 2011, Knippenberg et al. 2005, Weyand et al. 2012). This aspect will be covered more detailed in chapter 2.6.

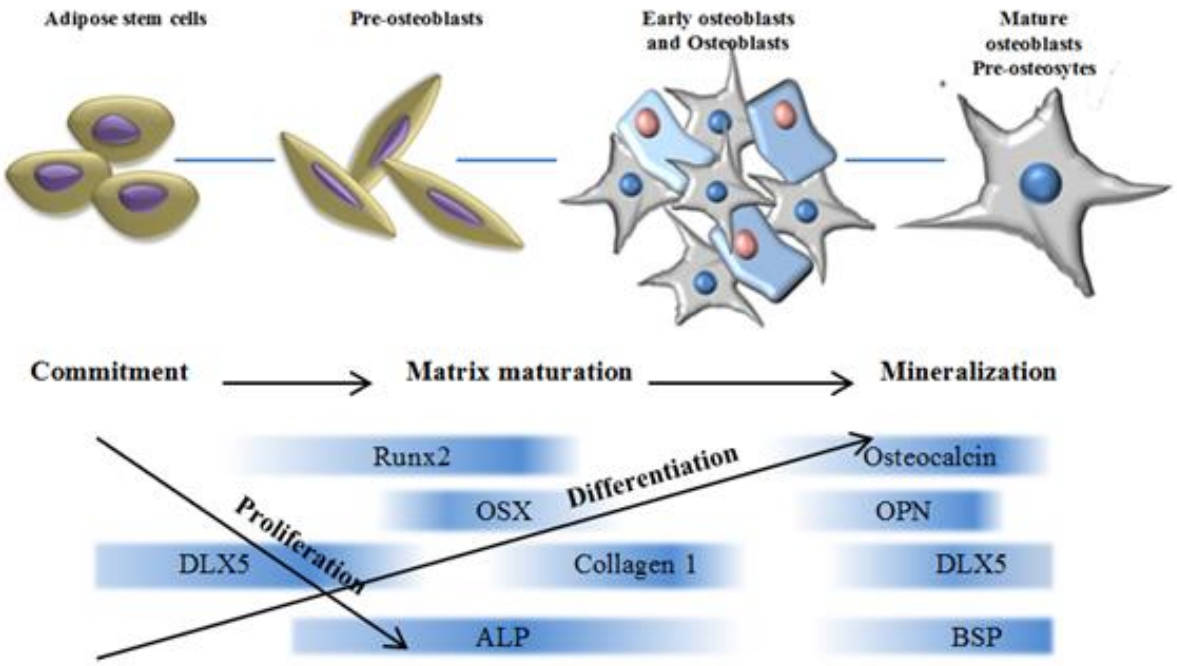


Figure 3 The osteogenic differentiation process and expression pattern of osteogenic markers. After proliferative phase cell start to differentiate and matrix maturation begins.

Osteoblast specific Runt-related transcription factor 2 (Runx2) is a key factor triggering early stage osteogenic differentiation. Other important factor related to osteogenic differentiation is osterix (OSX), acting directly downstream of Runx2 (Long 2011). Both Runx2 and OSX knockout mice have shown osteoblast differentiation arrest leading to the lack of bone development (Komori et al. 1997, Nakashima et al. 2002). The expression of many osteogenic

marker genes like osteocalcin, collagen type Iα1 (Collagen 1, COLL1), alkaline phosphatase (ALP), and osteopontin (OPN) is Runx2 expression dependent or related to Runx2 expression (Long 2011). In the matrix maturation phase after early proliferative phase of osteogenic differentiation the collagenous ECM starts to form and the level of the ALP activity peaks (Lian and Stein 1995). Collagen 1 rich ECM works as a platform for mineralization in the last phase of osteogenesis when osteopontin and osteocalcin, proteins associated with the mineralized matrix are expressed (Lian and Stein 1995). However, Runx2 synergize with many factors regulating its actions in osteogenic pathway. For instance, distal-less homeobox 5 (Dlx5) member of homeobox protein family (Msx1, Msx2, Dlx5, Dlx6) is essential for normal ossification and Dlx5 is known to activate the expression of Runx2 and bone markers as bone sialo protein (BSP) and osteocalcin, whereas other homeobox protein MSX2 possess opposite transcriptional properties and function as repressor of osteoblast marker gene expression (Holleville et al. 2007). Most of the early and late markers of osteoblast differentiation could be a direct target of Dlx5 (Holleville et al. 2007).

2.5.3 Cross-talk between adipogenic and osteogenic fate

In the level of transcriptional regulation, the osteogenic and adipogenic commitment might be more closely tied than generally expected. In bone marrow, the differentiation of osteoblasts and adipocytes is contrary regulated (Baek and Baek 2013). This regulation occurs mainly through signaling cross-talk between main transcriptional factors PPARγ and Runx2 (Baek and Baek 2013). The mitogen-activated protein kinase (MAPK) pathway (aka ERK/MAP pathway) plays an important role in this reciprocal control of adipo- and osteogenesis since both Runx2 and PPARγ are regulated by MAPK-dependent phosphorylation (Ge et al. 2009, van Beekum et al. 2009, Xiao et al. 2000). Important members of the MAPKs include extracellular regulated protein kinase (ERK), which has also revealed to be an essential molecule for mechanotransduction (Simmons et al. 2003). ERK phosphorylates Runx2 on serine residues which are required for Runx2-dependent transcription (Ge et al. 2009). These phosphorylation events have revealed to be necessary for the response of osteoblasts to several significant stimuli, including mechanical loading (Li et al. 2012, Ge et al. 2009). Similarly ERK-dependent phosphorylation inhibits transcriptional activity of PPAR γ and suppresses adipocyte differentiation (van Beekum et al. 2009). Recently Lee and co-workers have demonstrated in their study that PPARγ silencing not only inhibit adipogenesis, but enhances osteogenic differentiation of human adipose-derived mesenchymal stem cells (Lee et al. 2013).

2.6 3D culture

Cells naturally reside in three-dimensional niche, yet most studies on cell response to mechanical stimulation are based on two-dimensional (2D) planar, treated plastic surfaces. However, the behavior of the cells in two-dimensional culture differs from that in three-dimensional culture similar to *in vivo* environments (Antoine et al. 2014, Jung et al. 2016). Study conducted by Lin and co-workers (2014) indicates that cells cultured in 3D condition compared to 2D cultured cells had significantly better differentiation potential from day 14 to 21 for both adipogenic and osteogenic lineages when evaluated with Oil Red O and Alizarin Red staining (Lin et al. 2014). Further, it has also been shown that when soluble osteogenic inductive factors are included in the culture media, 3D collagen gel culture of hASC causes increased mRNA expression of bone markers Runx2, ALP, OPN, COLL1, osteonectin and mitogen-activated protein kinase 9 (MAPK9) compared with hASC cultured in 2D monolayer on tissue culture plastic (Gabbay et al. 2006).

There are three main features described in literature which may influence stromal cell fate and hence a controlled tissue morphogenesis in 3D cultures: (1) matrix stiffness and topography, (2) cell shape and (3) chemical and mechanical stimulation (Hwang et al. 2015, Mathieu and Loboa 2012, Huang et al. 2012, Mousavi and Doweidar 2015). Cell interactions with the extracellular matrix and physical signals such as matrix rigidity and mechanical stimuli have strong effects on cellular phenotype and tissue formation. Biochemical and mechanical cues from the ECM, sensed and transmitted mainly through integrins (Longhurst and Jennings 1998, Ingber 2003) determine the fate of human ASCs (Guilak et al. 2009, Takagi 2007).

Integrins are a protein family that comprises 18 α-subunits and 8 β-subunits in mammals (Hynes 2002) and form at least 24 heterodimers of one α- and one β-subunit (Legate et al. 2009). Upon binding to specific components of the ECM, integrins undergo a conformational change and form large aggregates, focal adhesions (FA) on the surface of the cells (Longhurst and Jennings 1998, Legate et al. 2009). Integrin associated intracellular protein complexes consequently control osteogenesis by modulating transduction signaling cascades such as phosphatidylinositol 3-kinase/Akt (PI3K-AKT), subgroups within the MAP kinase family (c-jun N-terminal or stress-activated protein kinases (JNK/SAPK), ERK/big MAP kinase 1 (BMK1), MAPK-ERK kinase (MEK) and the p38 group of protein kinases) and the Hippo pathway, an evolutionarily conserved pathway that controls tissue growth by the regulation of cell proliferation, differentiation and cell death (Hwang et al. 2015, Hynes 1992, Longhurst and Jennings 1998, Dupont 2016, Huang et al. 2012). Hwang et al. (2015)

suggested in their study that ECM stiffness regulates osteogenic differentiation through MAPK activation and specifically, a stiff hydrogel matrix stimulates osteogenic differentiation of MSCs, but inhibits adipogenic differentiation. ERK and JNK activity was significantly increased in cells cultured on a stiff hydrogel. Taken together, results suggested that ECM stiffness regulates MSC differentiation through ERK or JNK activation (Hwang et al. 2015).

Due to the fact that ECM impacts on cell proliferation, differentiation and other fates by specific molecular composition and mechanical properties, interactions between cells and the ECM is therefore crucial for *in vitro* expansion and differentiation of ASCs (Engler et al. 2006, Guilak et al. 2009).

2.7 Osteopromotive biomaterials in bone tissue engineering

Biomaterials used in bone tissue engineering scaffolds meet the certain demands as they serve as a substitutive ECM to organize cells and to present stimuli, which direct the growth and formation of a desired tissue (Drury and Mooney 2003, Rezwan et al. 2006). Scaffold material needs to be biocompatible (not being toxic, injurious, or physiologically reactive and not causing immunological rejection), biodegradable and porous by means of enabling the cell ingrowth, metabolism and vascularization of newly forming tissue (Rezwan et al. 2006). The degradation rate should match the formation of the new tissue and material stay durable enough to withstand the mechanical loading until newly formed tissue is sufficiently strong. Furthermore, the biomaterial should be degraded without leaving gaps or fissures in the tissue that encapsulates the stem cells (Neuss et al. 2008). Biomaterial used can potentially influence stem cell proliferation and differentiation in either positive or negative ways (Neuss et al. 2008). For example osteogenic differentiation of MSCs was preferably achieved in soft collagen-rich substrates, whereas adipogenic differentiation mostly occurred in stiff agarose-rich matrices (Duarte Campos et al. 2015).

Common materials used to substitute bone in clinic are metals like stainless steel and titanium. Although metals are strong enough to meet the mechanical challenges expected from bone they do not measure up requirements for biomaterials since being non-degradable. Today biomaterials used in bone TE can be roughly divided in three groups: synthetic polymers, natural polymers and bioceramics (Rezwan et al. 2006, Zanetti et al. 2013).

The majority of biodegradable synthetic polymers studied belong to the polyester family, which includes polyglycolides (PGA), polylactides (PLA) and polycaprolactone (PCL) (Vacanti and Langer 1999, Ashammakhi and Rokkanen 1997, Behravesh et al. 1999,

Middleton and Tipton 2000, Neuss et al. 2011). Disadvantages of PGA and PLA in TE applications are their poor biocompatibility, release of acidic degradation products, poor processability and loss of mechanical properties in very early stage during degradation (Kang et al. 2016, Ciapetti et al. 2003, Chen et al. 2016). Other synthetic polymers used in TE are hydrolytically degradable poly(ortho ester), -anhydrides, -acetals, -carbonates, -urethanes and polyphosphazenes and enzymatically degradable polyethers reviewed more closely by Ulery et al. 2011.

Natural polymers, peptides, proteins and polysaccharides are widely used in TE alone and combined with other materials. Archetypal naturally derived polymers include collagen, hyaluronic acid (HA), agarose, alginate, chitosan, fibrin, starch and gelatin (Drury and Mooney 2003). Scaffolds made from natural polymers can have different shapes, sizes and properties, which all can be modified with different compositions and processing of materials. As a matter of fact, poor qualities of both synthetic and natural polymer materials can be improved and enhanced bone-like characteristics can be obtained with co-polymerization and with usage of hybrid and composite materials. For example ceramics like hydroxyapatite (Neuendorf et al. 2008) and tricalcium phosphate (TCP) (Ehrenfried et al. 2008) have used to improve biocompatibility and mechanical properties of polymers used in bone tissue engineering as well as to neutralize acidic by products of polyester degradation (Ehrenfried et al. 2008). Surface reactive, amorphous bioactive glasses have also been in interest in past years due their ability to support and enhance the growth and differentiation of osteoblasts and as well as hASCs (Ardeshirylajimi et al. 2015, Haimi et al. 2009, Ojansivu et al. 2015)

A variety of synthetic and naturally derived materials may be used to form hydrogels for tissue engineering scaffolds (Drury and Mooney 2003, Rezwan et al. 2006). In this section a brief overview on hydrogels is given with a focus on natural collagen and HA hydrogels. The chemistry, gelling conditions, degradation modes and recent studies conducted of both are described below.

2.7.1 Hydrogels

The interest of hydrogels in bone tissue engineering has rose over the past years due to more sophisticate materials and processing methods. Hydrogels from different sources have ability to homogeneously encapsulate cells and hence enable research in three-dimensional surroundings, similar to *in vivo* environments (Alonso et al. 2008, Castillo Diaz et al. 2016, DeVolder and Kong 2012). Hydrogels are also considered to be good transducers of mechanical loading (Steinmetz et al. 2015).

Hydrogels are water-swollen polymeric networks, usually consisting of crosslinked hydrophilic polymer chains, which are either natural, synthetic or natural/synthetic hybrid polymers in origin and can swell but do not dissolve in water (Slaughter et al. 2009, Drury and Mooney 2003). Synthetic hydrogels are attractive materials for the TE because their controllable and reproducible properties. For example, synthetic polymers can be produced with desired molecular weights, block structures, degradable linkages, and crosslinking modes (Drury and Mooney 2003). As for naturally derived hydrogel forming polymers have commonly been used in TE applications because they are either components of or have macromolecular properties similar to the natural ECM (Munarin et al. 2012). Alginate, hyaluronic acid, and collagen, followed by chitosan and fibrin, turned out to be the most frequently used natural-derived hydrogels, being exploited with promising results for several biologic tissues (Munarin et al. 2012).

Highly hydrated hydrogels *mimic* the chemical and physical environment of ECM and therefore are ideal cellular microenvironment for cell proliferation and differentiation (Slaughter et al. 2009). Most importantly, hydrogels have a similar microstructure to the ECM and thus provide good physical integration into the bone (Lewandowska-Lancucka et al. 2015). Furthermore, hydrogel materials generally exhibit good biocompatibility and high permeability for oxygen, nutrients and other water-soluble metabolites (El-Sherbiny and Yacoub 2013). Hence, hydrogels have been utilized as scaffold materials for drug and growth factor delivery (Chung et al. 2007, Cao et al. 2014, Hayashi et al. 2009), engineering tissue replacements (Alhadlaq and Mao 2003) and a variety of other *in vitro* and *in vivo* applications. Polymerization of natural hydrogels and novel synthetic hydrogels can be carried out under mild conditions and thus in the presence of living cells (Drury and Mooney 2003). This allows homogeneous seeding of cells throughout the scaffold materials and hydrogel formation *in situ*.

Hydrogels are classified into physical and chemical hydrogels based on their cross-linking mechanism (Caliari and Burdick 2016). Physical (noncovalent) crosslinks are formed through self-assembly: entangled chains, hydrogen bonding, hydrophobic interaction and crystallite formation (Caliari and Burdick 2016). Physical crosslinks may not be permanent junctions but they are sufficient enough to keep the hydrogel from dissolving in an aqueous media (Caliari and Burdick 2016). Alternatively, chemical crosslinks are permanent junctions formed by covalent bonds (Caliari and Burdick 2016). Hydrogel networks may include both permanent and semipermanent junctions. The crosslinks formed between polymer chains via various chemical bonds and physical interactions impact on the structural integrity of the

hydrogels (Drury and Mooney 2003). The type and degree of crosslinking influences many of the network properties, like mechanics, swelling, mesh size, degradation and transport of molecules (Drury and Mooney 2003, Caliari and Burdick 2016).

2.7.2 Collagen I hydrogels

Collagen is a natural, most abundant protein in mammalian tissues (25% of the total protein mass of most mammals) and the main component of natural ECM (Munarin et al. 2012). Therefore, collagen is an ideal candidate for biomedical applications to support *in vitro* stromal cell growth and differentiation. Kruger et al. (2011) analyzed the relative osteogenic potential of two 3D biomaterials, type I collagen and poly(L-lactide-co-glycolide) (PLGA), to support *in vitro* mineralization of human mesenchymal stem cells. Human MSC s (hMSCs) were seeded onto three-dimensional PLGA or type I collagen scaffolds; incubated in osteogenic media; and harvested at 1, 4, and 7 days. Results suggested that long-term mineralization occurs earlier and to a greater extent on type I collagen, highlighting collagen as a potential bone tissue engineering scaffold for hMSCs (Kruger et al. 2011). Mizuno and Kuboki (2001) cultured BMSCs three weeks with type I collagen and observed mineralized tissues. When they interrupted the collagen-integrin interaction by the addition of DGEA peptide to the culture, the expression of osteoblastic phenotypes of bone marrow cells was inhibited (Mizuno and Kuboki 2001). Their findings demonstrated that integrin interaction supported by collagen matrix is an important signal for the osteoblastic differentiation of bone marrow cells (Mizuno and Kuboki 2001).

At least 19 different types of collagen have been presented, but the basic structure of all collagens is composed of three polypeptide chains wrapped around one another to form a three-stranded rope-like structure (Drury and Mooney 2003). Collagen is naturally degraded by collagenase, and serine proteases secreted by cells reside in the engineered tissue (Drury and Mooney 2003). Collagen I is usually isolated from bovine or porcine skin or from rat tail tendon and hydrogel properties have demonstrated to be source dependent (Kreger et al. 2010). Acidic collagen hydrogel polymerizes both covalently in the presence of sodium hydroxide (NaOH) and physically at 37 C° (Duarte Campos et al. 2015). After polymerization hydrogel is composed of a loose meshwork of collagen fibers and fluid. More detailed information on collagen I extraction and fabrication parameters, the reader is directed to article by *Antoine et al*. (Antoine et al. 2014).

Although natural polymers have similar micro- and nanolength dimensions of the fibril native ECM, their main drawback is poor mechanical properties, such as low load-

bearing capacity, ease of deformability and degradation-related mechanical weakening (Hutmacher 2010, El-Fiqi et al. 2013). For example, collagen gels have tendency to slightly deform by partly losing their water content that helped to maintain a certain volume. Thus, collagen alone is not easily patterned in a desired 3D shape (Duarte Campos et al. 2015). Additionally, collagen hydrogels shrink to a considerable extent during the culture of cells, an effect which is enhanced over time, and when the cell population becomes substantially increased (El-Fiqi et al. 2013). Furthermore natural polymers often contain residual growth factors, undefined or non-quantified substances, and batch-to-batch variations make it difficult to compare and correlate work between different groups (Caliari and Burdick 2016, Hutmacher 2010). Mechanical frailty restricts the extended use of collagen hydrogels, especially in applications for hard tissue regeneration *in vivo* (El-Fiqi et al. 2013).

Collagen I hydrogel studies

Alonso and co-workers (2008) used diffusion chambers implanted subcutaneously in rats to study rat ASC (rASC) osteogenesis seeded in collagen I hydrogel *in vivo*. Four weeks after implantation, *in vivo* bone and cartilage formation was demonstrated in implants containing osteo-induced cells wrapped in the collagen gel. Their findings imply that collagen I may support the survival and expression of osteogenic phenotype in passaged rASC *in vivo* (Alonso et al. 2008). Hao et al (2008) reported that by using collagen I gel to suspend rASCs into porous PLGA-β-TCP scaffold, osteogenic differentiation of rASCs can be improved and homogeneous bone tissue can be successfully formed *in vivo* (Hao et al. 2008). Furthermore Hesse et al. (2010) addressed the questions whether primary rBMSCs can attach to and migrate into collagen type I hydrogels and if these cells can proliferate and differentiate along the osteoblastic lineage. Their results indicated that collagen type I is likely to allow host rBMSCs to attach to and to enter the hydrogel. Furthermore, hydrogels enable the successful initiation of all these cellular processes and almost all cells remained viable and proliferating while expressing a marker of the osteoblast lineage and thus undergo osteoblast differentiation (Hesse et al. 2010).

2.7.3 Hyaluronic acid hydrogels

Hyaluronic acid has shown to interact in a favorable manner *in vivo* and thus have been exploited as hydrogel scaffold materials for TE applications (Rezwan et al. 2006). It is the simplest glycosaminoglycan (GAG) found in nearly every mammalian tissue and fluid. HA is especially prevalent during wound healing and in the synovial fluid of joints (Drury and Mooney 2003).

Hyaluronic acid is a linear polysaccharide composed of a repeating disaccharide of (1–3) and (1–4)-linked β-d-glucuronic acid and N-acetyl-β-d-glucosamine units. Hydrogels of HA are formed by covalent crosslinking with hydrazide derivatives by esterification and by annealing. Additionally, HA has been combined with both collagen and alginate to form composite hydrogels. HA is naturally degraded by hyaluronidase, thus allowing cells in the body to regulate the clearance of the material in a localized manner (Drury and Mooney 2003).

Hyaluronic acid hydrogel studies

Hyaluronic acid, with ASCs or mixed population of cells containing ASCs, has been used in recent studies alone and combined with other biomaterials. Most of the studies with HA aim at cartilage regeneration (Dinescu et al. 2013, Wang et al. 2016, Wang et al. 2014, Wu et al. 2013), adipose tissue research (Chang et al. 2013, Fan et al. 2015, Korurer et al. 2014, Tan et al. 2009, Tharp et al. 2015) and wound care applications (Catanzano et al. 2015, McMahon et al. 2016). Some studies with HA hydrogels and ASCs aiming at osteogenesis has also been reported. Park and co-workers (2016) used catechol-functionalized hyaluronic acid (HA-CA) hydrogel to potentiate stem cell-mediated angiogenesis and osteogenesis in two tissue defect models: critical limb ischemia and critical-sized calvarial bone defect. Study indicated that HA-CA-hydrogels retained the angiogenic functionality of hASCs and supported osteogenic differentiation of hASCs (Park et al. 2016). CA functionalization was used for decrease the cytotoxicity of photo polymerized HA hydrogels to human adipose-derived stem cells (Park et al. 2016). Alternatively Bea et al. (2011) studied whether the photo-cured hyaluronic acid hydrogel had suitable properties for use as a scaffold for bone tissue regeneration. They described in their study the positive influences on *in vitro* (fibroblasts) and *in vivo* (rabbit model) osteogenesis of photo-cured HA hydrogels loaded with simvastatin (SIM) and suggests that the photo-cured hydrogel was well qualified for use as a scaffold on osteogenesis with or without SIM. The results also suggested that the HA hydrogel had almost no cytotoxicity toward fibroblasts (Bae et al. 2011).

2.8 Mechanical stimulation to enhance bone formation

The human skeleton is constantly exposed to mechanical loading. Bone forming and adaptation processes in the bone tissue occur through body's own mechanosensors. Primary mechanotransducer cells osteocytes and lining cells initiates the combined action of hormones and growth factors and promote osteoblast differentiation in response to a wide range of

mechanical stimuli, including vibration (Robling and Turner 2009, Thompson et al. 2014, Papachroni et al. 2009). Mechanical loading have been shown to act through numerous signaling pathways (insulin-like growth factor 1 (IGF-1), integrin-focal adhesion kinase (FAK), Rho/ROCK signaling, BMP activated non-Smad pathways and Wnt/β-catenin) to promote osteoblast activity (Long et al. 2011, Thompson et al. 2012, Lessey et al. 2012, Kopf et al. 2012, Robling 2013, Qin and Hu 2014). In all cases these pathways directly or indirectly interact with MAPK. For example, binding of IGF-I to its receptor in turn activates Ras/Raf/MEK/MAPK pathway (Long et al. 2011). Additionally, cell exposure to stretching or fluid flow shear stress activates integrin signaling followed by activation of FAK and MAPK (Thompson et al. 2012). Furthermore, the activation of Rho and Wnt signaling indirectly activate or are themselves activated by MAPK (Lessey et al. 2012, Robling 2013). Thus, activation of the MAPK pathway is a common loading-induced signal in bone that can stimulate osteogenesis and inhibit adipogenesis via regulation of Runx2 and PPARγ discussed earlier in chapter 1.4.3. Mechanotransduction a process where cells convert mechanical stimulus detected into electrochemical activity is briefly illustrated in Figure 4.

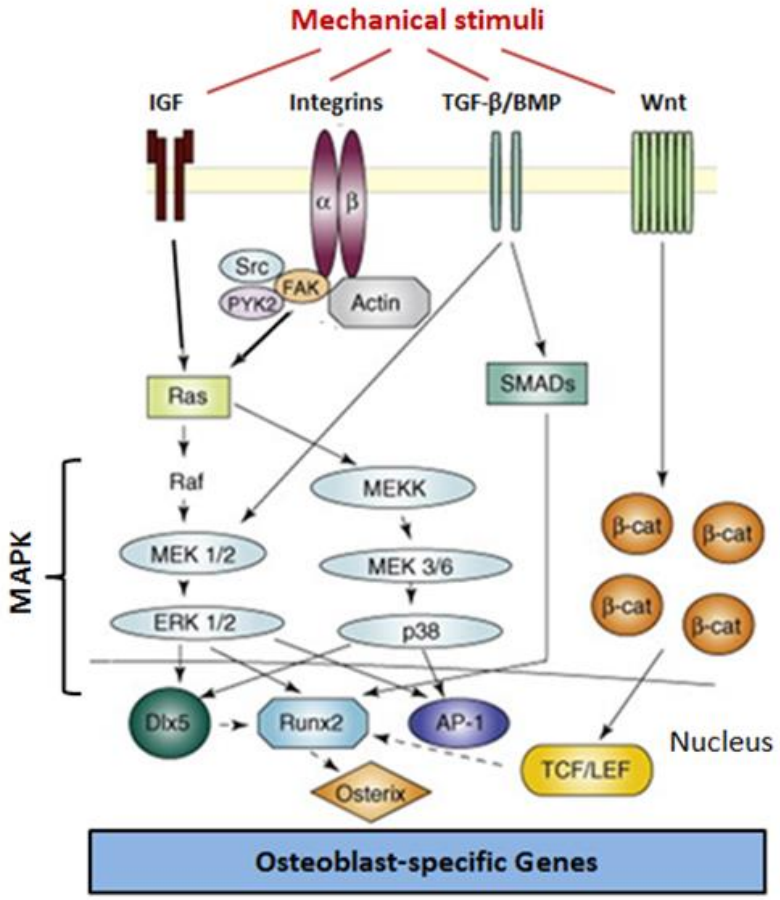


Figure 4 Mechanotransduction is a process where mechanosensitive cells translate mechanical forces generated outside cell into biochemical signals inside the cells. In turn, these signals can adjust cellular and extracellular structure. Cells sense these forces through intramembranous proteins like integrins and many different signaling pathways have proven to act to promote osteoblast activity. Important regulative pathway in osteogenesis activated by mechanical signals is MAPK. Image modified from (Papachroni et al. 2009).

2.9 Osteogenesis by using mechanical stimulation

Apart from the contractile force generated inside the cell, multiple studies have presented that mechanical force generated outside the tissue induces increased fluid flow in the bone matrix sensed by mechanosensitive cells and starts signaling cascade towards cell response and bone formation (Robling and Turner 2009). Mechanical signals have been shown to be inhibitory to fat and anabolic to bone *in vivo*, and to directly affect mesenchymal stem cell pools from which adipogenic and osteogenic precursors emerge (Sen et al. 2011). Since this thesis study deals with vibration stimulation and cellular effects induced by it, this part of the introduction focuses mostly on vibration induced signaling.

Vibration is the most common mechanical stimulus sensed by bone cells on a daily basis. LMHF vibration is a distinct force event that induces no or minimal membrane strain or fluid shear (Uzer et al. 2012). Vibration is a ubiquitous low-level mechanical stimulus that

can be applied at increased levels to human patients and this stimulus can also be replicated *in vitro* (Uzer et al. 2012). The main focus on this section will be on effects of mechanical stimulation on osteogenic differentiation, more specific in low magnitude high frequency (LMHF) mechanical stimulation which has been studied recently *in vivo* in clinical (Rubin et al. 2004, Gilsanz et al. 2006, Harrison et al. 2015, Kiel et al. 2015) and in animal tests (Park et al. 2012, Pagnotti et al. 2012, Sun et al. 2014, Hao et al. 2015, Judex et al. 2015, Chow et al. 2016, Gao et al. 2016, Jing et al. 2016, Xie et al. 2016, Marycz et al. 2016) as well as *in vitro* model systems (Tanaka et al. 2003, Pre et al. 2009, Kim et al. 2012, Park et al. 2012). Clinical trial test arrangements as well as results are variable, however so are the animal test and cell culture outcomes.

***In vivo* whole body vibration**

Clinical problems like non-union fractures and the increasing incidence of musculoskeletal system diseases such as osteoporosis have led to the investigation for safe, effective and simple therapies including whole body vibration (WBV). WBV is commonly performed to a subject standing on a vibrating plate that generates mechanical signals via vertical, horizontal, and/or pivotal accelerations to improve bone structure and muscle performance without requiring locomotion (Pel et al. 2009). Mechanical signals generated by LMHF vibration stimuli are considered to be capable to promote bone formation, increase bone strength, and even recover bone loss, but the underlying mechanism in cellular level is still poorly understood (Rubin et al. 2001, Judex and Rubin 2010, Ozcivici et al. 2010). However there is evidence that the mechanism of action is via effects of MSCs in musculoskeletal tissue (Pagnotti et al. 2012). The effects of various vibration protocols, as defined by their duration (exposure time), frequency (cycles per second, or Hz) and intensity (acceleration in g, where 1 g = Earth's gravitational force = 9.8 ms^{-2}) have been tested.

Most of the clinical studies have shown that LMHF applied whole body vibration positively influences bone mass at the range of frequency (20–90 Hz) or acceleration (0.1–0.5 g) for daily periods of 10 to 20 min, as well as two 10 minute periods per day (Judex and Rubin 2010, Rubin et al. 2004). However Harrison et al. (2015) tested skeleton responds of healthy pre-pubertal boys to WBV for 10 minutes in total, on both high (>2 g) or low (<1 g) magnitude vibration and resulted that the growing skeleton can respond quickly to vibration of either high or low magnitude (Harrison et al. 2015). There is also notable difference between outcomes in separate study groups. Gilsanz et al. (2006) reported that short exposure (10 minutes daily) to extremely low-level mechanical signals (30 Hz), several orders of

magnitude (0.3g) below that associated with hard exercise, increased bone and muscle mass in the weight-bearing skeleton of young adult females with low bone mineral density (BMD). On the other hand Kiel et al. (2015) observed in their study that the beneficial effects of WBV observed in previous studies with younger women may not occur to the same extent in elderly individuals.

***In vivo* animal models**

There is a broad range of *in vivo* animal studies with LMHF vibration from large animal models (sheep) to small rodent models. Similar parameters are used with animal studies as with human studies and outcome measures and study lengths vary greatly between different studies. Different conditions like fracture healing, osteoporosis and osteogenesis imperfecta models and aging have been studied with animals. In a large animal models like sheep the enhanced bone formation and improvement in bone structure as a consequence by LMHF vibration have been reported by Judex et al. (2003) and Rubin and the co-workers (2002). Various rodent studies on the effects of vibration stimulation have been conducted over the years. For example, Judex et al. (2005) studied vibration (45 Hz, 0.3g) for 10 minutes per day compared to hind limb unloading in murine model. They found increased bone formation and mineralization in the vibrated tibia and lower levels of expression for several osteogenic marker mRNAs in the disused conditions. The work suggested that vibration may prevent bone loss occurring during disuse. Furthermore, several groups have studied effects of LMHF vibration on the osteoporosis and aging murine models. For example Rubinacci et al. (2008) showed in their rat model that low-amplitude, high-frequency whole-body vibration (30 Hz, 0.6 g and 30 Hz, 3g 20 minutes per day) is anabolic to bone in ovariectomized animals (Rubinacci et al. 2008). Nevertheless the osteogenic potential was limited to the modelling of the bone cortex and depends on the amplitude of the vibration (Rubinacci et al. 2008). Animal studies with higher vibration parameters have also been done. Fracture healing of ovariectomized rats under vibration (90 Hz, 4g and 15 minutes per day) was studied by Stuermer et al. (2010). Their study demonstrated some beneficial effects of vibration on fracture healing, but they also find a reduction in the stiffness and yield load of the tibia after vibration and suggested that this might be due to the high acceleration used. They concluded that when using vibration therapy to improve fracture healing the conditions must be carefully executed in order to prevent harmful effects of vibration therapy.

***In vitro* cell studies**

Although the role of mechanical loading in maintaining the bone tissue is well established, the ideal vibration parameters and molecular mechanisms behind the adaptive responses are still riddle. Therefore human and animal studies have led to the investigation of vibration loading under *in vitro* models. Experiments conducted with animal (Tanaka et al. 2003, Dumas et al. 2010, Zhou et al. 2011, Kulkarni et al. 2013, Zhang et al. 2016) and human derived cells (Pre et al. 2009, Pre et al. 2011, Park et al. 2012, Gaston et al. 2012, Kim et al. 2012, Uzer et al. 2013, Zhang et al. 2015, Marycz et al. 2016) have indeed demonstrated that cells exposed to LMHF exhibit mechanosensitivity and show enhanced osteogenic marker incidence. Recent studies using human BMSCs (hBMSCs) or hASCs together with LMHF vibration for study osteogenesis are referred here. Also one study with HMHF stimulated ASCs is discussed (Tirkkonen et al. 2011). All studies referred have been carried out in 2D conditions where cells are typically seeded into a multi well plate (precoated with collagen if using osteocyte like cells) and plate is then attached to a loading rig, which may be commercially available or adapted in-house.

Kim et al. (2012) used hBMSCs from two different donors to investigate effects of LMHF vibration for osteogenic differentiation. They applied vibration at a variety of frequencies and accelerations (10, 20, 30 and 40 Hz and 0.1–0.6g in 0.1g increments) eventually choosing 0.3g and 30 Hz for their prime experiments. They observed that cell proliferation was affected by vibration in dose dependent manner with increases in cell number at higher frequencies. Also ALP activity was increased on days 7, 14 and 21 as well as mRNA for ALP, osteopontin and VEGF were upregulated in vibrated samples at day 7. Otherwise when cells were cultured in osteogenic media the calcium deposition increased in cultures vibrated, suggesting that LMHF vibration has increased osteogenesis. Interesting point noticed by Zhang et al. (2015) when investigating human periodontal ligament cells at large range of frequencies (10–60 Hz in 10 Hz increments and 90, 120, 150 and 180 Hz) was that osteogenic marker expression was also frequency dependent. Levels of COLL1, Runx2 and osterix increased at 40 and 50 Hz, while ALP activity was highest at 30 Hz. Osteocalcin expressions were higher at 40, 50, 60, 90 and 120 Hz.

Uzer et al. (2013) used LMHF vibration to apply shear stress to human ASCs. They looked at two frequencies (30 and 100 Hz) and magnitudes of 0.15, 1 and 2g for 30 minutes a day from 3 till 14 days. Cell number increased for all conditions over the first 3 d of culture, with the greatest increases seen for the lowest fluid shear measured (0.04 Pa). After 14 days mineralization was increased for all accelerations at 100 Hz and for the 30 Hz at 2g

acceleration. Also Pre et al. (2011) evaluated the effects of low amplitude, high frequency vibrations on the differentiation of hASCs toward bone tissue. hASCs were cultured in proliferative or osteogenic media and stimulated daily at 30Hz for 45 min for 28 days. They observed increased COLL1 and osteonectin expressions on day 14 and improved matrix calcification at day 21. However enhanced gene expression was lost by day 21 and increased mineralization compared to non-vibrated controls by day 28 (Pre et al. 2011). One study departed from the others used high magnitude (3g) and frequencies of 50 and 100 Hz to stimulate hASC towards bone-forming lineage (Tirkkonen et al. 2011). Cells were subjected to vibration loading for 3 hours per day for 1, 7 and 14 days. Both frequencies used induced increased ALP activity and Collagen production. Furthermore vibration HMHF vibration was noticed to inhibit adipocyte differentiation of hASCs, but it did not effect on cell number or viability.

3 AIM OF THE RESEARCH

Cells in the human body are in the influence of constant mechanical signals produced inside and outside the body and *in vivo* conditions are multidimensional instead of single directional. Hence, this study was made to translate the mechanical stimulation system from 2D to 3D in order to better mimic the natural environment of the cells. The specific aims of the study are listed below.

I. Finding a suitable hydrogel matrix, that should:

   a. Support the viability, attachment, and growth of hASCs cultured in basal medium.
   b. Allow or support the osteogenic differentiation of hASCs cultured in osteogenic medium and have sufficient mechanical properties to physically withstand the vibration loading stimulus.

II. Vibration loading and osteogenic differentiation of hASCs in the selected 3D hydrogel and comparison to static control.

4 MATERIALS AND METHODS

This study was carried out in two main steps: Phase II and Phase III. Hydrogel scaffold material selections (S I - II) was performed first. After material evaluation, three parallel vibration loading experiments (EXP I - III) with selected hydrogel were repeated. Phase I, the preparation step was repetitious part carried out before every round in Phases II and III. The general workflow of the study is presented in the Figure 5.

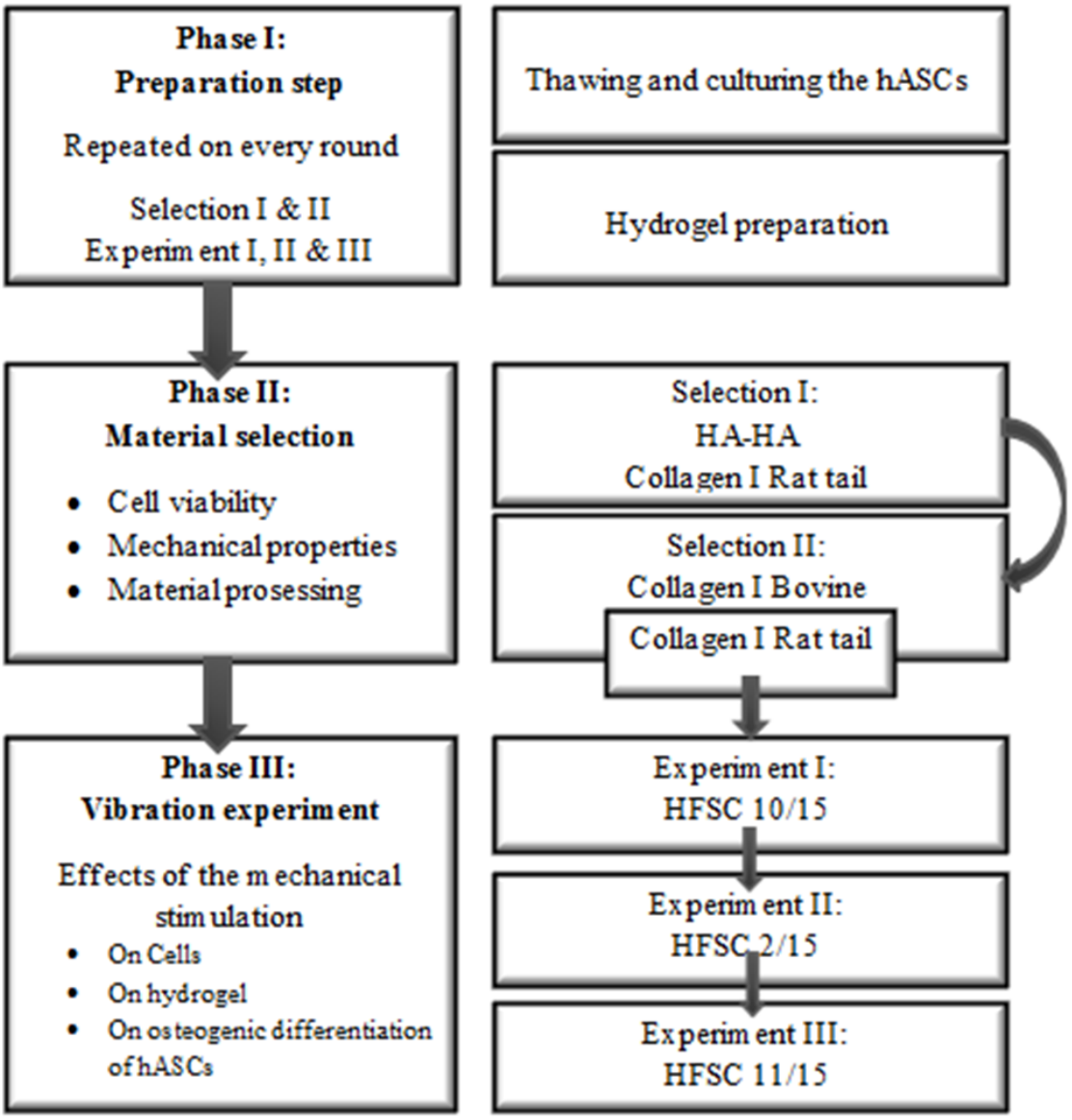


Figure 5 The workflow of the project. Study presented in this thesis was divided in three main phases I-III: Phase I was repeated in every round of the project, Phase II comprises two material selection studies and Phase III includes primary research; three parallel 3D vibration experiments with hASC (Human fat stem cells HFSC) isolated from three different donors.

4.1 Human adipose stem cells and their culture conditions

Stem cells used in this study were human adipose stem cells earlier isolated from adipose tissue samples from Tampere University Hospital, stored at cryogenic temperatures in dry phase freezer. All cells were collected with patient's consents.

In every experimentation step of this study the thawed hASCs were first maintained and expanded in T75 cm^2 polystyrene flasks (Nunc, Roskilde, Denmark) in basal medium (BM). BM consist in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Gibco® DMEM/F-12, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 5 % HS, 1 % 200 mM L-alanyl-L-glutamine (Gibco® GlutaMAX™, Thermo Fisher Scientific) and 1 % antibiotics (10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin, Thermo Fisher Scientific). When culturing, half of the medium was changed every other day until the cell cultures reached confluence. Confluent cultures were divided 1:2 or 1:3 depending how fast the cells were growing. Human ASCs used for the experimentations were in passages 2 - 3. Material tests (Phase II) were done with the cells in passage 2 and vibration experiments (phase III) when cells were in passage 3. Detaching the cells was accomplished by using recombinant cell-dissociation enzymes for mammalian cells (Gibco® TrypLE™ Select, Thermo Fisher Scientific)

Material testing was carried out using BM with the cell-seeded hydrogels. Bone cell differentiation was achieved in osteogenic medium (OM) when culturing cells with hydrogels in the vibration experiment. OM contained BM supplemented with 5 nM dexamethasone (Sigma-Aldrich, Saint Louis, MO, USA), 10 mM β-glycerophosphate (Sigma-Aldrich) and 250 µM L-ascorbic acid 2-phosphate (Sigma-Aldrich). In vibration experiments the OM for vibrated and static cells and BM for static controls was first added right after hydrogel gelation, changed after 1,5 hours, next day and then every other day. Cell-seeded hydrogels in BM were used as a control for osteogenic differentiation evaluation and static hydrogel cultures in OM as a control for studying the effects of the vibration.

4.2 Cell seeded hydrogels

Four different hydrogels were tested with hASCs in phase II: hyaluronan-based hydrazone crosslinked hydrogel (HA-HA hydrogel) mixed from two components (Aldehyde HALD 1, hydrazide HA-CDH) custom-made at Biomaterials and Tissue Engineering Group, Unit of Electronics and Communications Engineering, Tampere University of Technology; collagen type I from rat tail (rat-COL I 2mg/ml; Gibco® Life Technologies™, ThermoFisher Scientific); collagen type I from bovine (bov-COL I 2mg/ml; Gibco® Life Technologies™,

Thermo Fisher Scientific) and combination of hyaluronan-based hydrogel and rat-COL I hydrogel.

Before plating the cells in hydrogels, the following preparation phases were done: freeze-dried hyaluronan components HA-CDH (54.92 mg) and HALD 1 (54.66 mg) were both first mixed with 1.8 ml Dulbecco's phosphate-buffered saline (DPBS, Lonza, BioWhittaker, Verviers, Belgium) in 15 ml conical tubes (Falcon™ ThermoFisher Scientific). Falcon tubes were placed on a mixer in + 4C° cool room overnight. Next day the hyaluronan solvents were sterile filtered and HALD 1 component was measured out into the wells. hASCs were first mixed with the HA-CDH component and the cell-seeded mixture was added directly in the wells to form the 3D hydrogels. Collagen I mixture was prepared according to manufacturer's instructions. Work was done on ice and mixture's pH was determined with pH indicator paper (Whatman®, pH range 1–14, Camlab, Cambridge, United Kingdom) and adjusted before adding the cells. With combination hydrogels the hASCs were first added in the collagen I mixture and this blend was mixed with hyaluronan components by aforementioned manner. Phase II and III gelling protocols and materials are presented in Table 1.

Table 1 The gel reagents and amount of components as well as cell seeding densities of selection and vibration experiment phases. Gelation temperature with each gel type was 37 C° for 1 h.

Phase II	**Hydrogel materials/gel**	**Solvent**	**Cells/hydrogel**
Selection I	HALD 1 (100 µl)-HA-CDH (100µl)	PBS	1.9×10^5
	Rat-COL I (200 µl)	10 x PBS, 1M NaOH, Water	
	HALD 1 (80µl)-HA-CDH (80µl)–Rat-COL I (40µl)	PBS, 10 x PBS, 1M NaOH, Water	
Selection II	Rat-COL I (200µl)	10 x PBS, 1M NaOH, Water	1.9×10^5
	Bov-COL I (200µl)	10 x PBS, 1M NaOH, Water	
Phase III	**Hydrogel components/ gel**	**Solvent**	**Cells/hydrogel**
Experiment I–III	Rat-COL I (static+BM, static+OM, vibrated+OM)	10 x PBS, 1M NaOH, Water	1.0×10^5 (extras 0.5×10^5)

Cell seeding density used in phase II was decided to be 1.9×10^5 cells per hydrogel scaffold according to the previous tests made in the group and it was dependent on gel volume used (200 µl). Plate format, 48-well plate (Nunc) and the gel volume used were same in all

phase II and III experimentations. In Phase III the hASC seeding density was modified and decreased to half with 1.0 x 10^5 cells per well. With EXP II four extra gels were prepared with even lower cell density, 0.5 x 10^5 cells per gel. Blank gels were made before adding the cells in hydrogel mixtures. The prepared gels, with or without cells were incubated in 37 C° for an hour and after that 500µl medium was added on every gel.

In Phase II material selections the hASC hydrogels were cultured up to 10 (Selection I) and 15 (Selection II) days to evaluate gel properties, cell-gel interactions and cell viability by using light microscopy and live/dead staining protocol described below. In Phase III vibration experiments, after culturing hASCs in 3D hydrogel cultures 6–7 days (time point 1) and 12–13 days (time point 2), the adipose stem cell morphology, viability, number and osteogenic differentiation were characterized with methods presented next.

4.2.1 Cell morphology and viability

In phase II and III, the morphology of hASCs was observed by light microscopy. Light microscopy was used to verify the effects of hydrogel to the cells and also to observe changes in cell morphology during vibration.

Qualitative Live/dead cell viability assay (Invitrogen, Thermo Fisher Scientific) was used to evaluate the viability of the hASCs loaded in the hydrogels. The cell viability and attachment were assessed in Phase II material selections I and II and in Phase III experiments I and III (Figure 6). The method is based on fluorescent probes Calcein acetoxymethyl ester (Calcein-AM) and ethidium homodimer 1 (EthD-1). The former is a cell permeant dye which is converted in living cells by intracellular esterase activity to a green fluorescent calcein. Latter enters cells through damaged membrane and produces red color when binding nucleic acids.

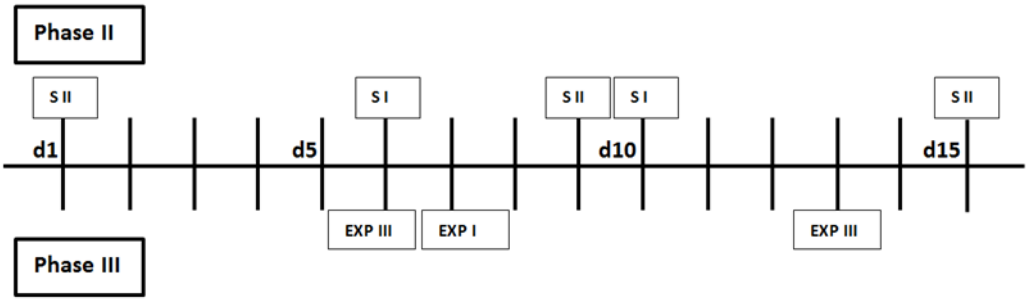


Figure 6 Timeline of the cell viability assays conducted in Phase II and III.

Briefly, in time points the culture medium was removed and hydrogel cultures were washed once with DBPS and incubated 45 mins at room temperature (RT) in the working solution containing 0,5 µM Calcein-AM and 0.0312 µM EthD-1 in PBS. Staining was

visualized and images of viable (green) and dead (red) cells were obtained by using a fluorescence microscope (Olympus IX51) with fluorescence optics. Adobe Photoshop version CS4 was used for picture editing.

4.2.2 Cell Number

The cell number was determined quantitatively with CyQUANT Cell Proliferation Assay Kit (CyQUANT® Life Technologies™, Thermo Fisher Scientific) according to the manufacturer's guide. The analysis is based on the amount of total DNA in the sample, as CyQUANT GR dye in working solution (WS) expresses fluorescence when bound to nucleic acids.

Quantification was performed for the samples collected at the two selected time points from every three vibration experiments. At the time points cell seeded hydrogel scaffolds were washed with DPBS and lysed with 0.1 % Triton-X 100 (Sigma-Aldrich) in sterile Eppendorf tubes and mechanically mashed with Pellet Pestle (Sigma-Aldrich) to get the cells out of the scaffolds. Cell lysates were frozen and stored in -70 C° until further use. When analyzed, three parallel 20µl samples of each lysate were pipetted onto a 96-MicroWell plate (Nunc) and 180µl WS added in every well. Fluorescence was measured with microplate reader (Victor 1420 Multilabel counter) at 480/520 nm.

4.2.3 Alkaline Phosphatase quantification

ALP enzyme can be used as a marker to characterize early osteogenic differentiation of hASCs. In this study the existing alkaline phosphatase was qualitatively illustrated by quantitative alkaline phosphatase assay (qALP). In the procedure yellow colored p-nitrophenol is cleaved from p-nitrophenol phosphate by ALP. The color intensity is proportional to the catalytic concentration of ALP in the sample.

The ALP activity was determined from the same Triton-X 100 cell lysates as for the analysis of DNA amount, described in Cell number-section above. After thawing, three parallel 20µl samples of each lysate was pipetted onto a 96-well PCR-plate (Nunc) and incubated with an alkaline buffer solution (2-amino-2-methyl propanol; 1,5 M, pH 10.3; Sigma Aldrich) and p-nitrophenol phosphate (Sigma Aldrich) for 15 min in 37 C°. Immediately after incubation the reaction was stopped by adding 50 µl NaOH (1 M, Sigma Aldrich) in every well. Samples were transferred onto a 96-MicroWell plate and the color intensity was determined with a microplate reader (Victor 1420 Multilabel counter) at 405 nm.

4.2.4 Osteoimage Mineralization assay

Osteoimage™ mineralization assay kit (Osteoimage, Lonza, Walkersville, MD, USA) was used to determine the mineralization in the vibrated and static hASC + OM cultures. Assay is based on the specific binding of the fluorescent staining reagent to the hydroxyapatite (HA) deposited by cells. Method was used as an alternative for typically used histochemical methods like Alizarin Red staining. Unlike Alizarin Red, that reacts with calcium portion of the phosphates Osteoimage method is HA specific and considered to be less complicate quantitative technique to explore osteogenic differentiation.

Before staining, gels were first washed once with PBS and then fixed with 4 % paraformaldehyde (PFA; Sigma Aldrich) 30 min in RT. After fixation samples were handled according to manufacturer's instruction for 48-well plate. *In vitro* mineralization was assessed quantitatively using a plate reader (Victor 1420) at 485/535 nm. Before fluorescent microscopy imaging Osteoimage buffer was removed and samples were furthermore stained with 4'6-diamidino-2-phenylindole (DAPI) diluted in distilled water (dH_2O; 1:2000). After 5 min dark incubation samples were washed three times with dH2O, the third wash was left in the wells and samples stored at +4C° for immunostaining.

4.2.5 Immunocytochemical staining

An antibody-based method immunocytochemical staining (ICC) was used to detect specific proteins in the samples. Focal adhesion formation and cytoskeletal reorganization were analyzed by immunofluorescent staining of F-actin and vinculin (focal adhesion-specific protein). For analyzing osteogenic differentiation, immunofluorescent staining for late osteogenic marker protein osteocalcin was also made. Result was imaged by the fluorescence microscope (Olympus IX51).

Staining was performed in Phase III for gels obtained from EXP I, II and III at the second time point. The vinculin staining was done for the same samples as Osteoimage and DAPI staining presented earlier. F-actin and osteocalcin staining samples were processed manner expound next. Briefly, the samples were first washed twice with DPBS and fixed with 0.2 % Triton-X 100 in 4 % PFA. After 15 min incubation in RT samples were kept overnight at +4 C°. Blocking the samples with 1% Bovine serum albumin (BSA; Sigma Aldrich) in PBS was carried out on the next day (3h, RT), followed by primary antibody incubation. The primary antibodies diluted in 1 % BSA in PBS are presented in Table 2. The secondary antibodies and phalloidin-TRITC (Sigma Aldrich) 1:500 were added on samples after overnight incubation in +4C°. Before adding the secondary antibodies, the samples were

washed four times with PBS with 3 min incubation at each round. Secondary antibodies and dilution used are also listed in Table 2.

Table 2 Primary and secondary antibodies used in immunostainings

Staining	Primary antibody	Dilution	Manufacturer
Vinculin	Vinculin ABfinity™ Recombinant Rabbit Monoclonal Antibody	1:100	ThermoFisher Scientific
Osteocalcin	Anti-osteocalcin (IgG) Mouse Monoclonal Antibody	1:100	Life technologies
	Secondary antibody		
Vinculin	Donkey Anti-rabbit IgG Alexa Red 680	1:800	ThermoFisher Scientific
Osteocalcin	Donkey Anti-mouse IgG Alexa Green 480	1:500	ThermoFisher Scientific

After 45 min incubation (dark, RT) three more 3 min wash rounds with PBS were done and third washing solution on vinculin samples was left in wells for imaging and on osteocalcin stained samples last washing solution was supplemented with DAPI (1:2000). 5 more min incubation in RT for osteocalcin samples and similar washing rounds as with Osteoimage samples after DAPI add were performed. Staining was visualized with a fluorescence microscope (Olympus IX51) and the images were edited using Adobe Photoshop version CS4.

4.2.6 Quantitative real-time PCR

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was used to analyze expression of known genes in samples of interest. In this study the levels of Human aP2 gene and different osteogenic genes; ALP, Runx2A, Dlx5 and COLL1 mRNA transcripts relative to the level of human Ribosomal Protein Lateral Stalk Subunit P0 (RPLP0) transcripts as a control were determined by quantitative RT-PCR. The total RNA was isolated from the experiment I–III cells in two time points and first strand cDNA was synthesized from the total RNA.

Briefly, at the time points cell seeded hydrogel scaffolds were once washed with DPBS and lysed with RA1 buffer (supplemented with 1% β-mercaptoethanol) of the NucleoSpin® RNA Isolation Kit (Macherey-Nagel, Düren, Germany) in sterile Eppendorf tubes and mechanically mashed with Pellet Pestle (Sigma-Aldrich) to get the cells out of the scaffolds. Cell lysates were frozen and stored in -70 C° until further use. Total RNA was isolated from the samples after few weeks freezing period using the RNA Isolation Kit according to the user's manual. Isolated RNA was diluted in 50µl RNase free H_2O and RNA concentration were determined by means of NanoDrop 1000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) at 260/280 nm. Total RNA was reversely transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit from Applied

Biosystems™ (ThermoFisher Scientific). qPCR analysis was performed by using probes for all investigated targets. Detailed list with the primer sequences and accession numbers are presented in Table 3.

Table 3 Primer sequences and accession numbers used in RT-PCR

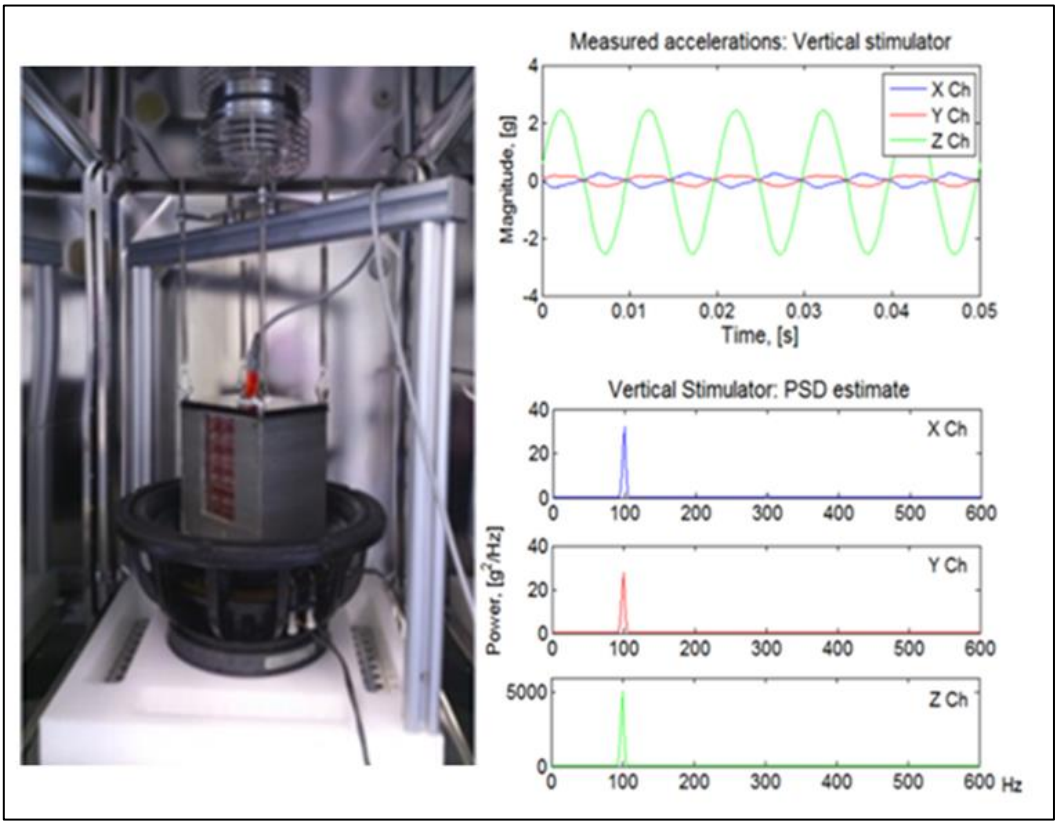
Gene		Sequences	Product size (Bp)	Accession number
RPLP0	Forward Reverse	5'-AAT CTC CAG GGG CAC CAT T-3' 5'-CGC TGG CTC CCA CTT TGT-3'	70	NM_001002
aP2	Forward Reverse	5'-GGTGGTGGAATGCGTCATG-3' 5'-CAACGTCCCTTGGCTTATGC-3'	71	NM_001442
ALP	Forward Reverse	5'-CCC CCG TGG CAA CTC TAT CT-3' 5'-GAT GGC AGT GAA GGG CTT CTT-3'	73	NM_000478.4
Runx2A	Forward Reverse	5'-CTTCATTCGCCTCACAAACAAC-3' 5'-TCCTCCTGGAGAAAGTTTGCA-3'	62	NM_001024630.3
Dlx5	Forward Reverse	5'-ACCATCCGTCTCAGGAATCG-3' 5'-CCCCCGTAGGGCTGTAGTAGT-3'	75	NM_005221.5
COLL1	Forward Reverse	5'-CCA GAA GAA CTG GTA CAT CAG CAA-3' 5'-CGC CAT ACT CGA ACT GGA ATC-3'	94	NM_0008

The qRT-PCR mixture comprise 50 ng cDNA, 300nM forward and reverse primers and SYBR Green PCR Master Mix (SYBR® Select Master Mix, Applied Biosystems™, ThermoFisher Scientific). Each sample was analyzed as duplicates. The reaction was accomplished with an AbiPrism 7300 Sequence Detection System (Applied Biosystems) and the data managed with 7300 system software program and with Microsoft Excel 2010. The data was normalized to the expression of RPLP0 housekeeping gene. The expression levels of the investigated genes in Static and vibrated OM cultures were compared with expression levels of static BM control cultures at first time point.

4.3 Mechanical stimulation of the cells

Vertical vibration loading was used to mechanically stimulate hASCs in Phase III. Rat tail collagen I hydrogel selected based on the initial cell culture screening in Phase II was used in the vibration loading study. The vibration loading experiment was repeated thrice by using cells from three donors. A Controllable vertical vibration for cell plates was achieved with HMHF vibration loading device placed inside a cell culture incubator (Figure 7A). Device has been designed and built at the Biomedical engineering department, Tampere University of Technology, Finland. The Appliance is composed of a sound-producing subwoofer driven by microcontroller based signal source. The sine wave signal used was amplified by audio amplifier to produce desired g-force. A peak acceleration of 2.5 g was achieved by adjusting the output amplitude in 600mV.

Vibration loading was started 24 h after plating the hASC seeded gels. Cell culture plates were superimposed on the subwoofer in the rack especially designed for the well plates used. The acceleration of vibrations was measured at the top centre of the rack with the ADXL321 accelerometer (Analogue Devices inc., MA, USA). Cells were vibrated with a peak acceleration of 2.5 g, using a sine wave at 100 Hz with an effective vibration period of 30 minutes with 150 min break. Device carried out five vibration periods in 24 hour with last break duration 510 minutes. Same stimulation period was applied every day till first and second time point. Medium change for the cells was performed every other day during the last cycle break. The static OM and BM control cells were cultured similarly under standard cell culture conditions (37 C°, 5 % CO_2). Data from reliability of the acceleration and frequency was collected with LabView programmed standalone software (Figure 7B).



A **B**

Figure 7 A) Mechanical stimulation for the cells was achieved with the vibration loading device inside the cell culture incubator. B) The data from stimulation was collected and reliability and optimal function after every stimulation start checked with standalone software.

5 RESULTS

5.1 Phase II - Material selection

5.1.1 Hydrogel behavior during and after plating

In hydrogel preparation phase feasibility of the hydrogel processing was evaluated. HA-HA and HA-HA-COL I gels were somewhat complex to produce; it was impossible to mix the cells homogenously into the gel before full gelation, which then also resulted in uneven gel surface. In addition, filling the whole well was not achieved with all samples. Furthermore, the pH of the hyaluronic acid gels could not be measured or modified. Gelation of the collagen I gels was not as rapid as with HA-gels. Hence, fabrication process enabled measuring and adjusting the pH of the mixture before adding the cells.

All hASC hydrogel cultures at hydrogel selection phase were monitored for cell growth and morphology by light microscopy and live/dead cell viability assay. The culture conditions for all cell seeded hydrogels were the same, thus the biomaterial effect on the cells was easy to evaluate. The pH decrease indicated by the yellow color in growth medium was also evaluated and it was more rapid in gels containing hyaluronic acid components than with collagen gels. Resulting, that BM on the cell cultures had to be changed almost immediately after plating and then every day to maintain the pH of the culture media acceptable. The phenomenon was most likely the result of dissolution of the acid products of the hydrogel in the growth medium rather than more rapid cell division in these gels. It is noteworthy that all the combination hydrogels were completely dissolved in the growth medium after two days of culturing and all hyaluronic gels lost their mass radically during the culturing period. Both collagen gel types were observed to maintain their shape and mass longer than hyaluronic acid gels. The bovine origin gels started to shrank on the ninth day from plating and rat-COL I gels after few days from that. Hence rat-COL I gels incline to resist the growing cell population better.

5.1.2 Cell morphology was dependent on hydrogel used

Light microscopy was used to evaluate cell seeding density and regularity right after plating and changes in cell morphology during culturing. Since only basic medium was used for testing the cell hydrogel interactions, no osteogenic behavior was expected. Mainly changes in cell morphology and count were evaluated to observe the action of hydrogel to the cell viability.

Cell morphology with all hydrogels was round right after plating as expected. As mentioned earlier, the cells seeded in HA-hydrogels were not spread evenly due to challenges in the mixing phase. Furthermore, cells seemed to dwindle during culturing. In the first hydrogel selection, there were also problems with collagen-seeded cells: after a few days of culturing the cells appeared like swollen and problems with pH were suspected. Microscopy images from day 6 hydrogel selection I are presented below in Figure 8. Light microscopy pictures from HA-COL I gels were not taken because all gels dissolved during the culturing.

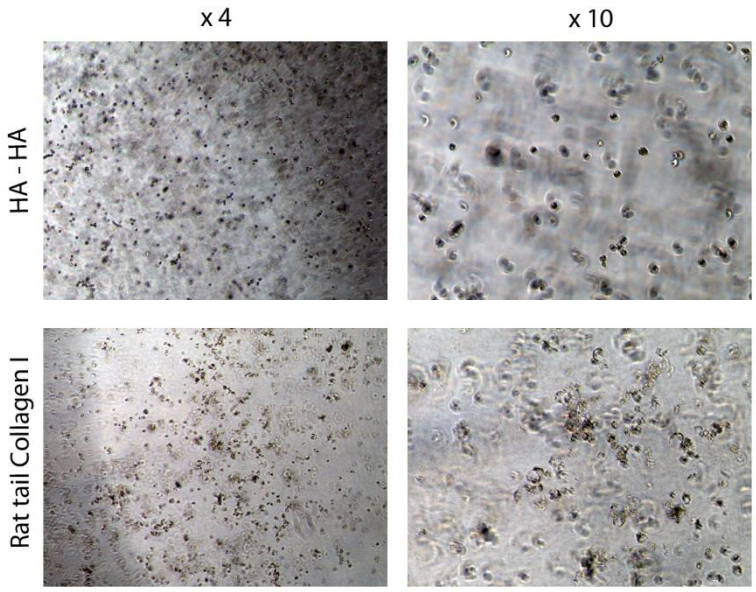


Figure 8 Round shaped cells can be seen in light microscopy pictures from day 6 gels in selection I. When observing cells in collagen I gels more carefully some unexpected vagueness in cell morphology was noticed. This was thought to be due to problems with pH levels in gel. (Marks x 4, x 10 stands for magnifications of objective lenses).

Despite the problems with the rat-COL I gels in selection I, this hydrogel material was selected to the next round since HA-gels were challenging to process, uniform cell seeding density was not accomplished, gels lost their mass and cell viability was compromised. Additionally pH regulation was not possible during the processing.

In the gel preparation process of selection II phase, a close attention to pH control was paid. After the collagen mixtures were produced the pH level was adjusted before adding the cells. Controlling the pH levels was beneficial and cells looked viable under the light microscopy during the culture period (Figure 9).

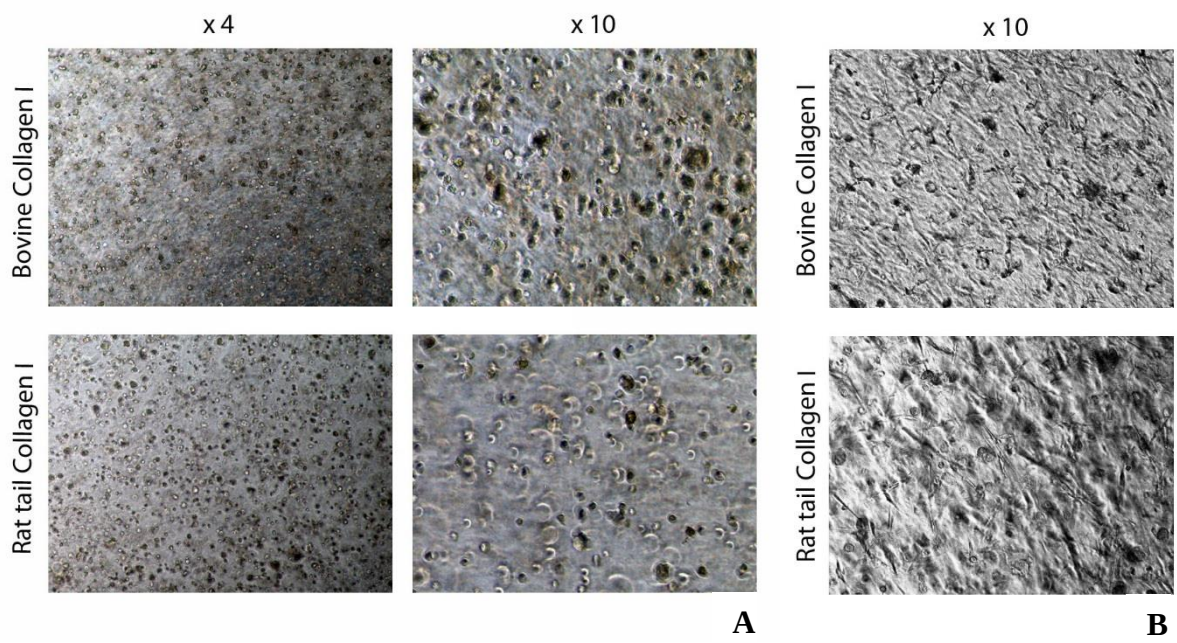


Figure 9 A) A lot of round shaped cells can be seen in Hydrogel selection II gels on day 1 B) On day 5 the cell morphology distinct greatly from cell morphology seen in selection I. Cells are spread and more spindle shaped.

5.1.3 Live/dead cell viability assay for evaluating hASC viability on biomaterials

Viability of the cells was also observed qualitatively by Live/dead assay in the biomaterial selection study. Good cytocompatibility was observed with both collagen materials, but with the hyaluronic acid gels the evaluation was overly challenging due to poor gel quality. Only a very few live cells and some dead ones could be seen in HA gels at the first time point and even less in the second time point (Figure 10).

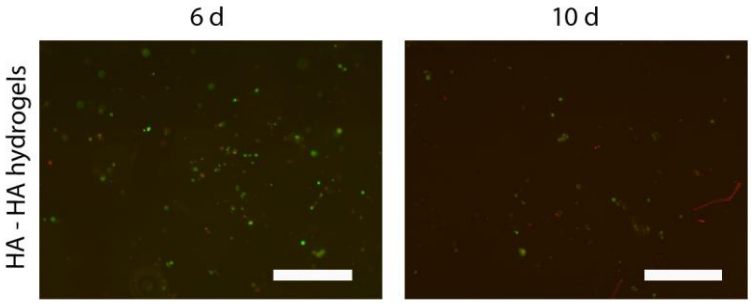


Figure 10 Live/dead assay performed for HA-HA hydrogels after 6 and 10 days culturing in hydrogel selection I revealed only a few viable cells and with relation to that lot of dead cells. Cell population also decreased over time. Scale 500 µm.

Instead, a lot of viable, live cells and only a few dead cells could be detected in the hASC-seeded collagens at all time points. Furthermore, the number of the cells appeared to increase over the time in both collagen type hydrogels as evaluated qualitatively (Figure 11).

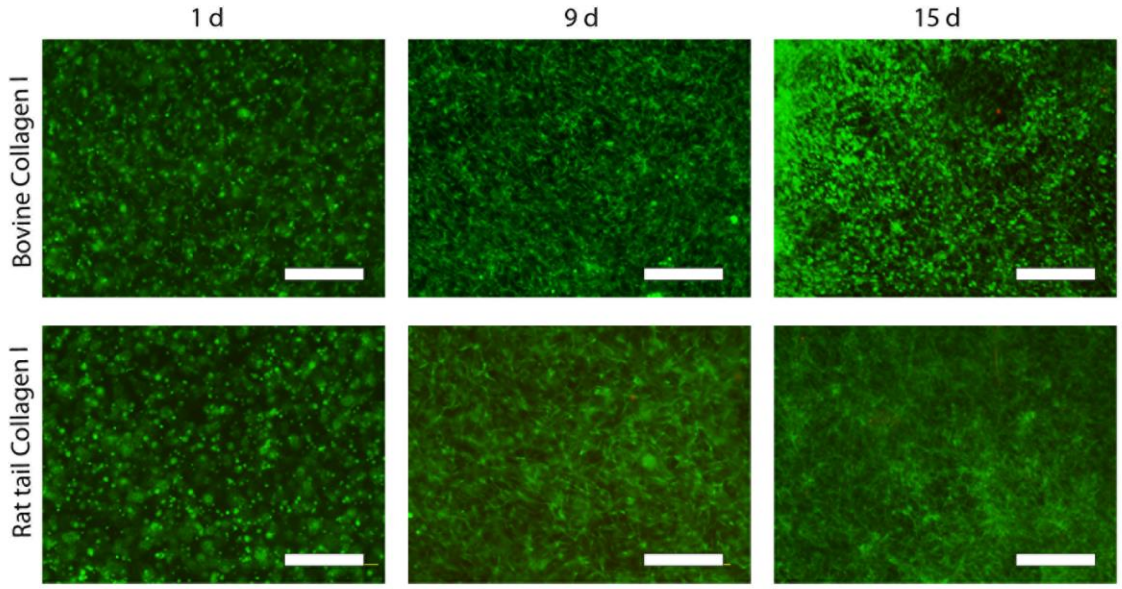


Figure 11 Viability of hASCs after 1, 9 and 15 days of culture. Live/dead assay performed to collagen I hydrogels in selection II indicated high cytocompatibility of collagen I from both origins. Scale 500 µm.

5.2 Summary of the Phase II

Hydrogel selection was done in two steps as described above. From the selection I the rat-COL I was taken to the next selection step due to the reliable fabrication process and better properties than hydrogels based on hyaluronic acid. Selection II was done between collagens from bovine and rat origin. Although, it seemed that bov-COL I and rat-COL I gels supported cell growth and viability equally, the ability to resist the pulling forces of the growing cell population favored the rat-COL I. Under these circumstances collagen I from rat tail was selected for vibration loading experiment since the mechanical strength was one important selection ground for the hydrogel. Other significant outcome from this phase was the importance of the pH monitoring before adding cells in the gel mixture to eschew unfavorable culture conditions.

5.3 Phase III - Vibration experiments

5.3.1 Cell proliferation affects greatly on the hydrogel properties on hASC-cultures

In phase III vibration loading experiments hASCs from three different donors were seeded in hydrogels containing collagen I extracted from rat tail. As it was previously discovered, hydrogel's capability to resist pulling forces created by growing cell population is restricted. To avoid gel detaching the cell count employed in phase III was halved from the original used

in phase II. Variation between the proliferation rates of separate donor cells was observed during the cell culturing period. Especially hASCs (HFSC 2/15) in EXP II showed extremely fast increase in cell count during phase I resulting two times higher cell density than EXP I hASCs and almost five times higher than EXP III hASCs in two weeks expanding period. The same trend with the proliferation rates was observed after cell seeding.

Experiments I, II and III demonstrated that the cell proliferation speed affected on hydrogels: hydrogels in EXP I shrank more rapidly than EXP III gels in which cells grew slowest in the phase I. Moreover, the gels in EXP II detached from the well walls and shrank rampantly just in few days. Four extra gels made with lower cell density maintained the physical shape only few days longer. Live/dead cell viability assay, Osteoimage Mineralization assay or ICC staining could not be performed for EXP II gels, because of the shrinkage. A similar trend with shrinkage was detected with vibrated OM and static OM gels. Vibrated OM gels shrank faster than static OM gels with all cell lines. There was also a difference between the static OM and static BM gels—BM gels survived without shrinking almost throughout the whole experiment. The pH decrease indicated by yellow color in medium was also more rapid in vibrated OM cultures than in static OM cultures. The same outcome was also observed with parallel experiments: EXP II medium was rapidly changing its color and with EXP III medium color change was slower than with other experiments. The phenomenon was most likely the result of rapid cell division and higher cell number in experiment II.

5.3.2 Effects of vibration loading on cell viability and morphology

Light microscopy was used to observe cell morphology, viability and amount during vibration loading. With experiments I and III the morphology between vibrated and static OM cultured cells was estimated on day 5 (Figure 12). Corresponding comparison in EXP II was not possible to accomplish because all gels were contracted.

There were no distinct differences in morphology or cell count between the two groups evaluated i.e. vibrated and static cells. Both conditions supported spindle-shape morphology and gels were evenly crowded with the cells. Although, it seemed that there were cells in multiple layers it was challenging to estimate the cell morphology inside the gels. Thus, it might be that cells were more spread out on the top of the gels and bottom of the wells than inside the gels.

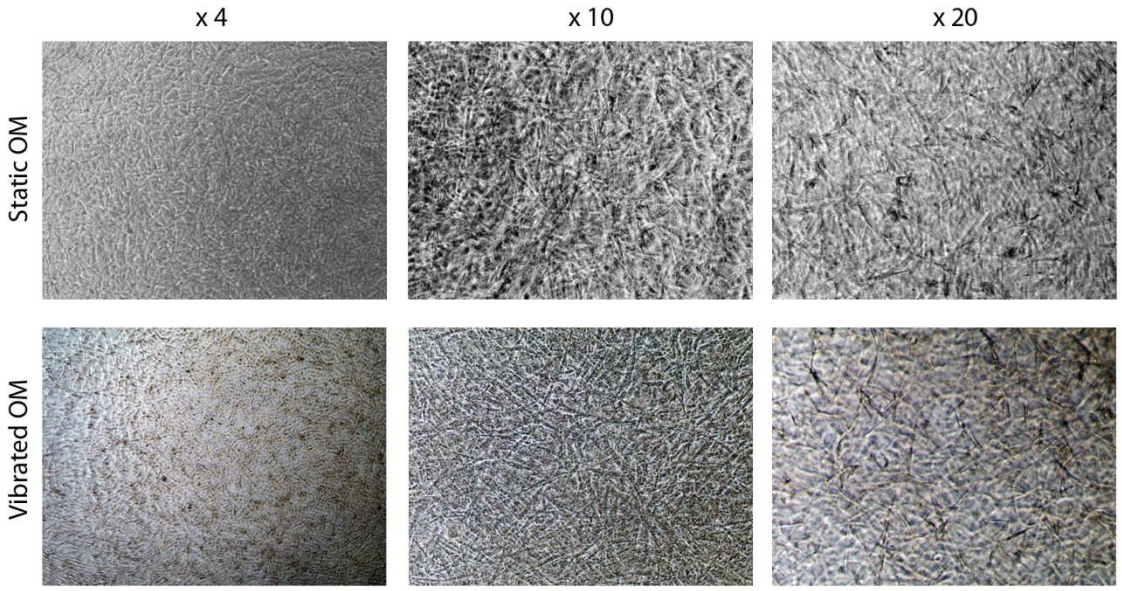


Figure 12 Light microscope pictures from day 5 static and the vibration-stimulated cells in experiment III.

Viability of the cells was also observed qualitatively by live/dead assay in the vibration loading studies. The assay was performed only for experiment I gels in first time point and experiment III gels in both time points, since the quality of the dwarfish gels ruled out the test (Figure 13).

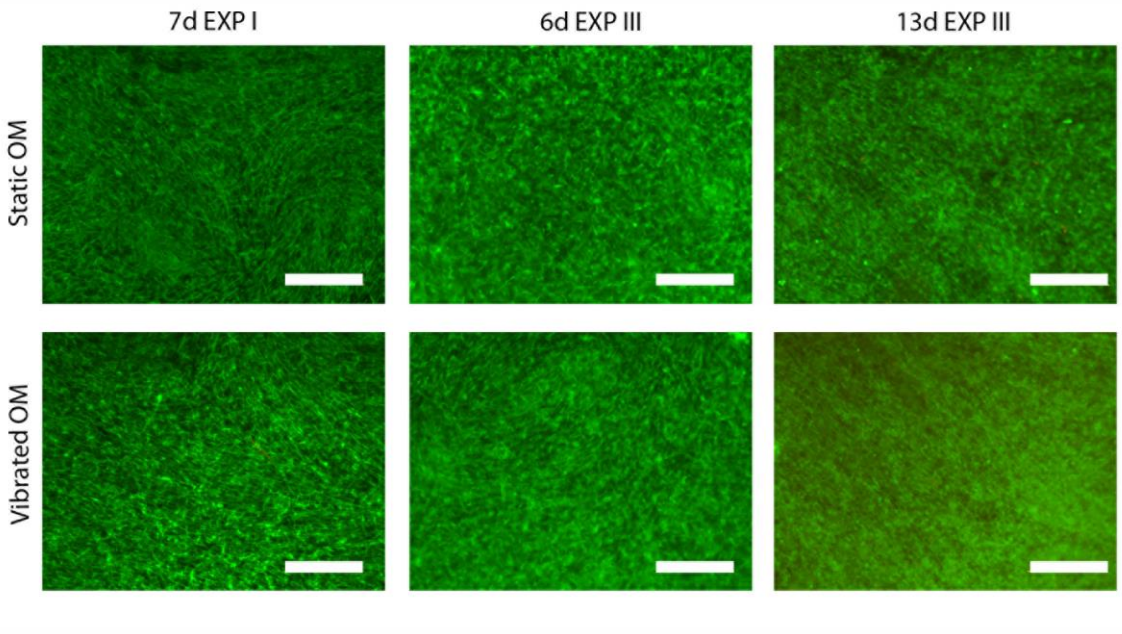


Figure 13 Fluorescence microscope images of the static and the vibration-stimulated (100 Hz) hASCs in OM at 7 (EXP I) and 6 and 13 day (EXP II) time points as visualized by live/dead staining of live cells (green) and dead cells (red). Vibration loading did not affect the viability of the hASCs. Scale bar 500 µm.

The live/dead assay supported the results of the light microscopy observation. A lot of viable, cells were observed with negligible number of dead cells. The number of the viable cells increased over time in both conditions when evaluated qualitatively and no notable difference with viability of the hASCs was observed.

Thus, vibration loading did not greatly affect, neither negatively or positively, morphology or viability of the vibrated cells, although some difference in the appearance of the cell cultures could be seen. In the vibrated cultures, there were some cloudy effect on top of the gels, which hindered focusing both light and fluorescence microscope. Moreover, when estimating pictures from live/dead assay, slightly more crowded gels were observed in vibrated cultures.

5.3.3 Detecting cell attachment and osteogenic differentiation of the cells

Immunostaining was conducted for evaluating focal adhesion formation and cytoskeletal reorganization in the experiments I and III, also staining for late osteogenic marker protein osteocalcin was made. F-actin, osteocalcin and nuclei staining were conducted in the same samples (Figure 14) and the staining for focal adhesion protein vinculin was made using the same gels as for Osteoimage Mineralization assay.

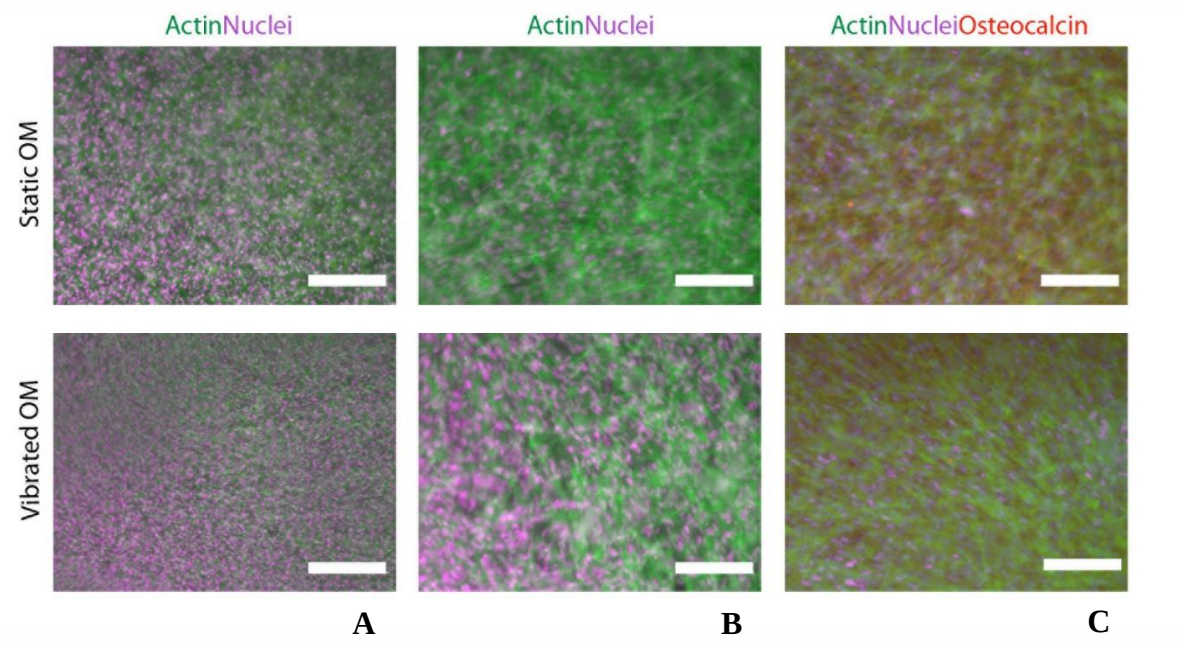


Figure 14 A) Phalloidin and DAPI staining for cell morphology carried out for experiment III cells after 13 days culturing. Scale 500 µm. B) Actin cytoskeleton formation more closely. Scale 200 µm. C) Phalloidin, DAPI and osteocalcin ICC. Scale 200 µm

DAPI ICC demonstrated the same fact as seen in previous studies: there was great amount of cells in both cultures, even more in vibrated ones. Furthermore, well-orientated actin microfilament formation was observed in both culture types with both cell lines and there was

no observed difference between the samples. Closer inspection of the filament formation was challenging due to the cells growing in multiple layers. Regardless, the orientation of the vibrated OM and static BM cell's actin filaments are presented in Figure 14 B, but the cell cytoskeletons on focus are probably growing on the top of the gels or bottom of the well. Actin fiber orientation was similar in both static and vibrated cells.

Osteogenic differentiation of hASCs in both cultures was evaluated by staining osteocalcin in the samples (Figure 14 C). Faint staining can be observed in both experiment III culture types. There was no detectable staining in experiment I gels. Additionally the effect of vibration loading on hASC osteogenic differentiation was evaluated by visualizing mineralization of the collagen cultures. In experiment I there was great difference between the static and the vibrated cultures in the mineralization levels (Figure 15).

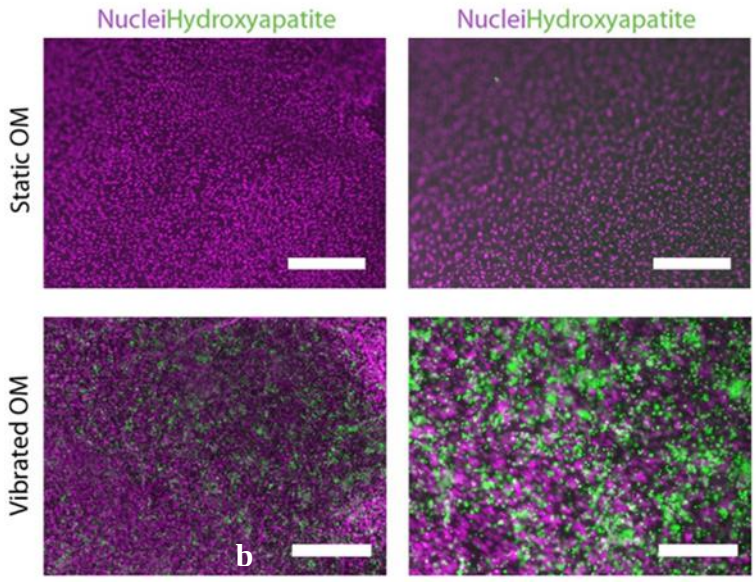


Figure 15 Osteoimage Mineralization assay for two parallel EXP I static OM cell cultures in second time point demonstrated no hydroxyapatite deposition. Scale 500µm. Notable higher degree of mineralization was detected with vibrated OM cell cultures in second time point. Scale a) 500 µm b) 200 µm

Basically no hydroxyapatite was detected in static OM cultures with experiment I, but with experiment III both culture conditions showed same outcome: lot of mineral particles in both culture conditions (Figure 16).

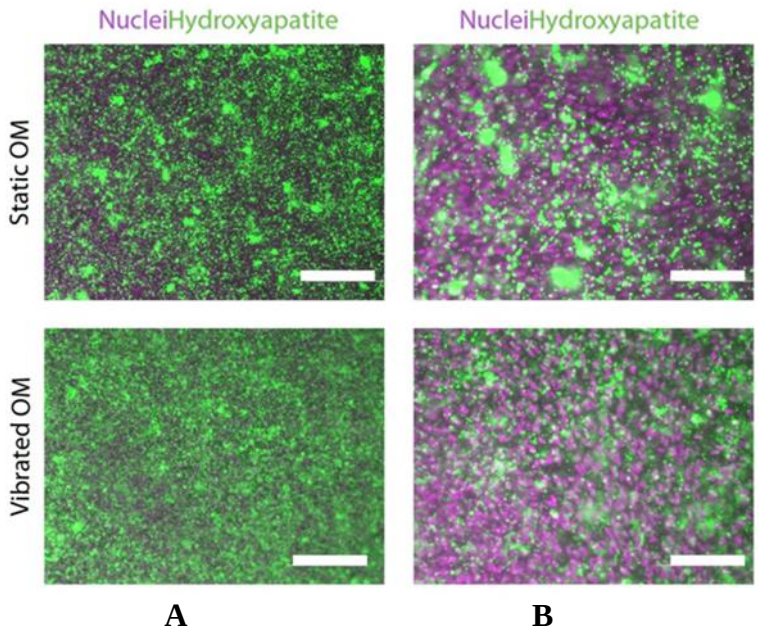


Figure 16 In Osteoimage Mineralization assay for experiment III gels in second time point there was not similar difference in outcomes between static OM and vibrated OM samples than with experiment I. Scale A) 500 µm B) 200 µm

5.3.4 CyQUANT Cell Proliferation Assay to evaluate proliferation

The influence of vibration loading on cell number was detected quantitatively with CyQUANT cell proliferation assay (Figure 17)

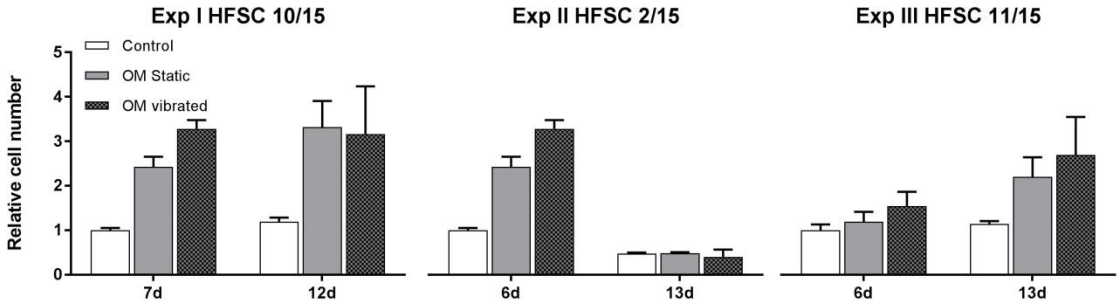


Figure 17 Quantitative analysis of relative cell number at time points I and II in experiments I–III. The results are relative to the first time point control.

In all experiments I–III, in the first time point the cell proliferation in vibrated cultures was higher than in the control BM and static OM cultures. In second time points 12 and 13 days after culturing slightly decreased number of vibrated cells was observed with experiments I, instead the static OM cell count was increased. In this stage of culturing all the vibrated gels in experiment I were shrunk and the result could be dependent on that. Very low cell count was also registered in all experiment II gels in all conditions at that point which bear out the hypothesis that shrinkage of the gels affects the result. Furthermore, in experiment III the cell

proliferation was higher in both OM conditions compared to control in both time points and vibrated cells demonstrated substantial proliferation capacity than static OM cells thru culturing period. Notable is that only experiment III gels survived the whole survey time without significant shrinkage.

5.3.5 Osteogenic differentiation

Alkaline Phosphatase quantification

The effect of vibration loading on hASC osteogenic differentiation was evaluated with qALP. As seen in Figure 18, the ALP activity increased in vibrated cells after 6 or 7 days culturing in experiments I and III. In second time point only experiment III vibrated cells demonstrated increased ALP activity. With experiment II cells the ALP activity in both culturing conditions in first time point was even lower than control and showed almost no alteration after 13 days of culturing. As previously, also in this assay it was clear that the shrinkage of the gels impacted on the result.

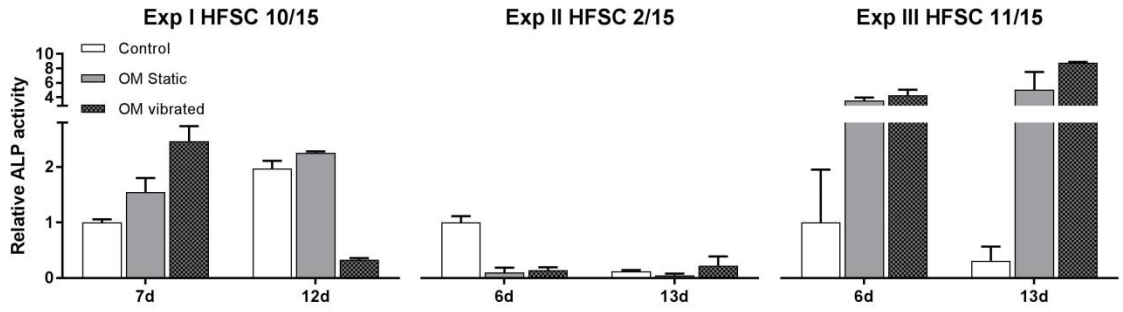


Figure 18 Relative ALP activity at time points I and II in experiments I–III. Notice the different scale in Experiment III. The results are relative to first time point control.

When the ALP activity is normalized to the corresponding cell amount, no significant or consistent increase in ALP activity with vibrated cell was detected (Figure 19). Normalized ALP activity in experiment I was even lower than control in both OM groups in both time points. In experiment III the ALP activity in static OM and vibrated OM cultures was almost the same, i.e. vibration appears not to have any effect on osteogenic differentiation in this manner. Thus, the elevated ALP activity in mechanically stimulated hASC lines 10/15 and 11/15 in comparison to static cells was probably due to increased proliferation.

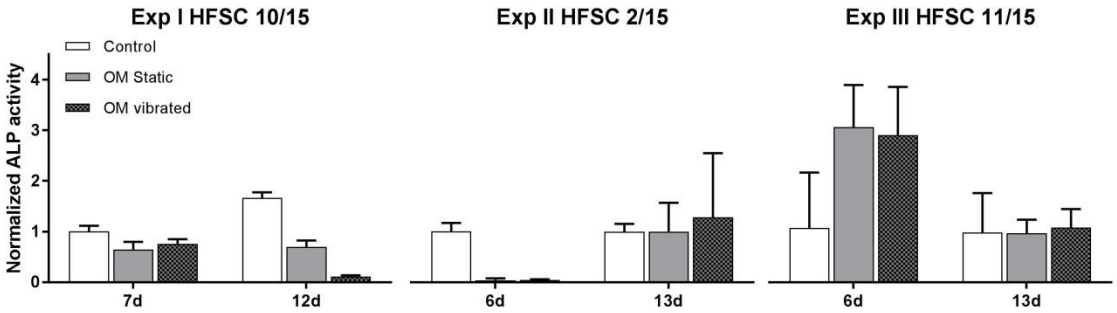


Figure 19 Quantitative ALP activity normalized with the cell amount from CyQUANT at time points I and II in experiments I–III. The results are relative to first time point control.

Quantitative real-time PCR

Expressions of five selected genes were studied with qRT-PCR and effects of vibration loading on the degree of these gene expression levels was analyzed.

In OM conditions relative expression of adipogenic marker aP2 was elevated with all cell lines compared to control (Figure 20A). Vibrated cells showed up to ten threshold increases in expression levels in experiment III after 13 days culturing, while in experiment I the expression was only slightly raised. In experiment II, aP2 expression was also explicitly higher in vibrated cells. A notable finding was the uniformity of ALP gene expression (Figure 20B) and normalized ALP activity levels presented in previous chapter. As with ALP activity the gene expression levels were also higher or about the same in static OM cultures than with vibrated cells. Hence vibration had no effect or it even decreased relative expression of ALP when cultured in osteogenic medium. When studying the expression of the Runx2A gene (Figure 20C) the same outcome was seen as with other osteogenic marker ALP. Static OM cultures showed higher expression levels compared to control and also to vibrated cells. Only exception was detected in experiment III at the first time point, where mechanically stimulated cells had higher expression levels of Runx2A than static OM cells. Third osteogenic marker to investigate was DLX5 (Figure 20D). All the vibrated cell lines showed increasing levels of DLX5 expression compared to control and static OM cells. With the experiments II and III the gene expression was notably higher in the second time point with vibrated cells than with cultures without mechanical stimulation. Also collagen 1 production of hASCs was analyzed to examine the *in vitro* formation of organic bone matrix (Figure 20E). The results were almost entirely consistent with ALP and Runx2A results: Vibration decreased the expression levels compared to static OM cultures, except at second time point in experiment II.

As a summary, vibration loading had increased adipogenic aP2 and osteogenic DLX5 gene expression in two out of three experiments, yet with other osteogenic genes this type of behavior was not seen, but rather opposite. Shrinkage of the gels did not appear to affect the gene expression analysis. The greatest differences between the cell lines were in expression levels which could be due to donor variation, proliferation rate and stage or shrinkage of the gels.

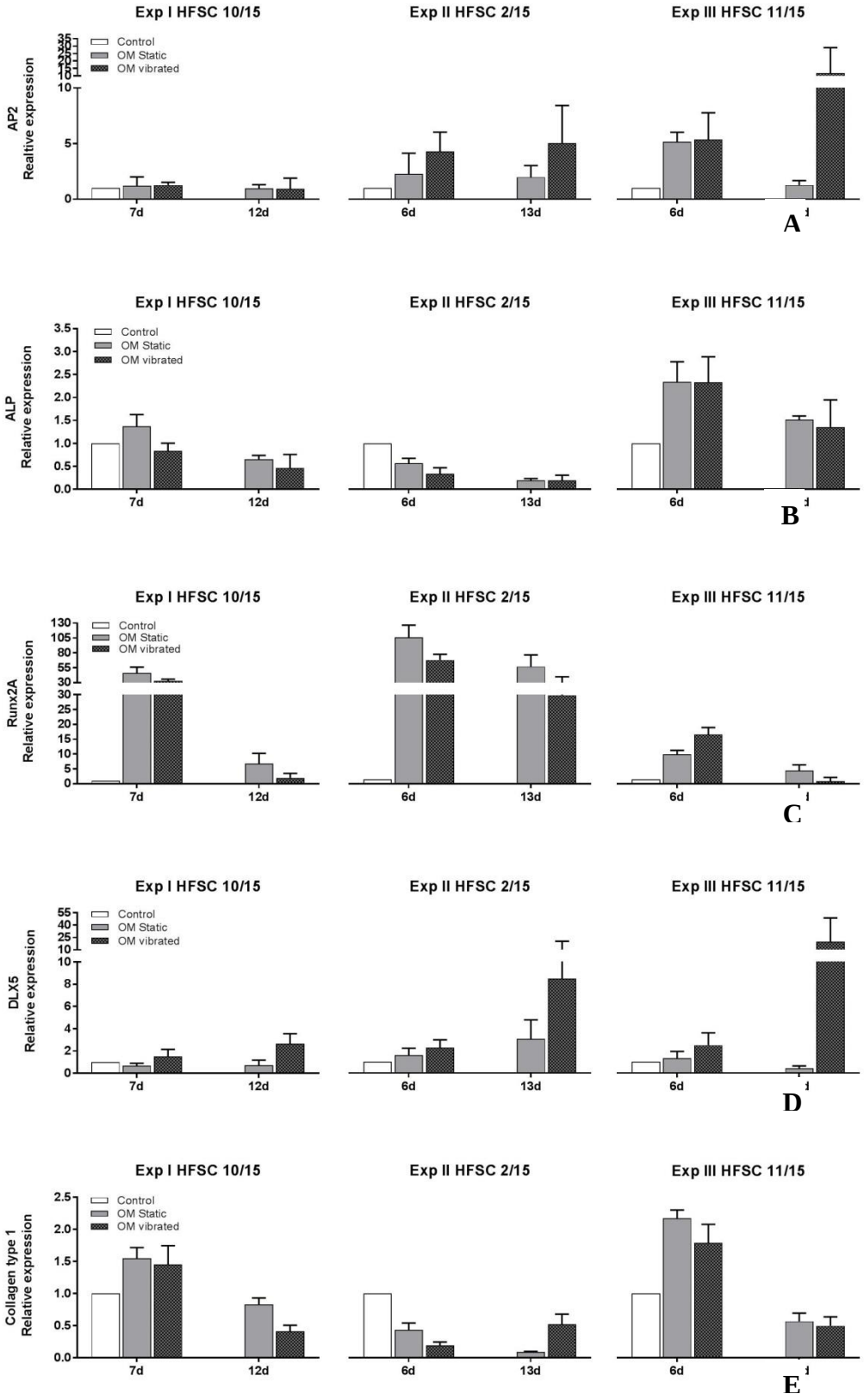


Figure 20 Relative expression of AP2 (A), ALP (B), Runx2A (C), DLX5 (D) and collagen type I (E). All results were normalized to the expression of human RPLP0 housekeeping gene and standardized to the static BM control.

6 DISCUSSION

The study presented here was a continuation for the work published by Tirkkonen et al. (2011). Like most of the recent studies discussed in the literature review, also the study by Tirkkonen and co-workers was conducted under vibration in the 2D culture. The specific aim of this thesis study was to translate the concept into 3D environment. Due to the pioneering nature of this project the aim was to find a suitable material to support the cell proliferation and osteogenic differentiation and sustain the forces generated by vibration loading, and furthermore, test how the vibration parameters used with 2D cultures would work with the cells in 3D medium. In this study the effects of mechanical stimulation by HMHF vibration loading on hASCs from three different donors, seeded in collagen I hydrogels was observed.

6.1 Rat tail collagen I hydrogels supported hASC viability in all culture conditions

When, studying cells with biomaterials an essential requirement for the material is the ability to support the cell viability. Furthermore, demand for the hydrogel used in these experiments was the capability to transfer mechanical loading to the cells and yet to withstand destructive forces generated by vibration and growing cell population. Cell viability in the biomaterials was first estimated in phase II in basal medium and collagen I from rat tail was selected for the phase III owing to its superior properties compared with the other tested hydrogels.

In phase II cells were cultured in static conditions in BM to assess the hASC response to the surrounding matrix. Based on morphology and viability of the cells, there was a problem with cytocompatibility of the rat-COL I hydrogels in the first material selection phase. In second selection such a problem was not seen, neither with the experiments I - III in phase III. This could be due to the batch to batch variation that may be occasionally a problem with the natural polymers purified from the animal origin (Caliari and Burdick 2016). Perhaps, the most likely explanation is an anomaly in the fabrication process. While preparation temperature and collagen purity as well as monomer-oligomer proportion and protein completeness all have great effect on gel properties, is pH probably the most important parameter concerning cell wellbeing (Antoine et al. 2014). The pH of the gels was not measured in the first material selection experiment, but because of the complication detected in cell morphology it was gauged in future in all preparation steps. Since slightly too acidic pH (6) was often detected immediately after mixing the gel components, it is essential for the cell viability that the pH of the collagen mixture is checked and adjusted before adding the cells.

In phase III, when evaluating the effects of the vibration loading on cell viability and morphology the following things were noted: both OM conditions, vibrated and static, supported spindle-shape morphology of the evenly spread viable cells in the gels, but slightly higher cells number was observed in the vibrated samples when estimating pictures from live/dead assay. This result was supported by quantitative CyQUANT Cell Proliferation Assay. A well-orientated actin microfilament formation in cell cytoskeleton with ICC staining was observed in both culture types. However, 3D culturing made it difficult to decipher cell morphology and cytoskeleton formation especially with time, due to the multi-layered cultivation, gel shrinkage and plausible ECM production, which made visual analysis impossible.

In conclusion, hASCs from all donor lines, in all culture conditions stayed viable in rat-COL I when fabrication parameters, especially pH, were checked properly. Vibration did not seem to have an impact on cell viability or morphology, but it did affect significantly the cell proliferation in increasing manner with all cell lines.

6.2 Hydrogel shrinkage compromised research frame

Another concern apart from the batch to batch variation and fabrication parameters used in the gelling process a possible change in the mechanical properties of the hydrogels. When evaluating mechanical properties and behavior of collagen I hydrogels the following was concluded: cell proliferation rate and further the increasing cell number affects the hydrogel properties. Collagen I hydrogels detached from the well walls and shrank more rapidly if the cell proliferation rate was high. Static BM control gels which showed lowest relative cell count in CyQUANT Cell Proliferation Assay with all cell lines at first time point also shrank least, hardly at all, whereas vibrated cell with higher proliferation speed and cell count shrank faster. Control gels without cells did not shrink at all. The gels in second time points of the experiments were more or less shrank and with experiment II gel shrinkage started even before first time point. However as observed with live/dead staining with experiment I and III cells, the hydrogels supported survival of the cells even after gels started to shrink. Effects of the substantial reduction in gel volumes on the cell viability with experiment II gels could not be evaluated with staining, but results of the CyQUANT analysis imply that massive shrinkage causes considerable decrease in the cell count aka cell death. Shrinkage is likely to change the mechanical properties of the gels, i.e. increase the stiffness of the gels. This could in part have an effect on the cells, partly by a) how the increased ECM stiffness induces

signals in the cells and b) the increased stiffness affects how the vibrational stimulus travels in the hydrogel.

Several studies have reported that collagen I as a biomaterial can enhance osteogenic differentiation of cells (Alonso et al. 2008, Hao et al. 2008, Hesse et al. 2010). Thus it is considered as a beneficial matrix for this type of experiments conducted. However surface stiffness and topography have a significant effect on both proliferation and differentiation of mesenchymal cells (Mathieu and Loboa 2012, Hwang et al. 2015). When hydrogels shrank during culturing, stiffness and topography, hence as a consequence cell behavior changes. One way to try to improve collagen I hydrogels to withstand pulling forces generated is partially dehydrated plastic compressed (PC) collagens where fibril density of the gels is increased in a controlled manner (Cheema and Brown 2013). In the PC process the excess fluid trapped in the initial hyper hydrated gel is forced out, reducing the fluid content by 100 - 200 -fold, typically from 99.8% to 85% - 90%, which is comparable to native tissues (Cheema and Brown 2013). Moreover, adding mechanically stronger components to hydrogel matrix is used. To overcome mechanical problems concerning the use of collagen I hydrogels in bone tissue engineering El-Fiqi et al. (2013) demonstrated the effective role of inorganic, mesoporous structured bioactive glass nanoparticles (mBGn) in significantly improving the physicochemical and mechanical properties of collagen I hydrogel for bone tissue engineering purpose. They incorporated the amine functional group surface modified mBGn into the collagen hydrogels, enabling a positive-charged surface. As a result, the hydrolytic and enzymatic degradation of the Col-mBGn hydrogels were slowed and the mechanical properties of the hydrogels, specifically the resistance to loading as well as the stiffness, significantly increased, as assessed by a dynamic mechanical analysis. While the collagen hydrogel showed extensive shrinkage during a few days of culture, the shrinkage of the mBGn-added hydrogel was substantially reduced and had no observable shrinkage over 21 days. Also MSCs cultivated within the collagen-mBGn hydrogels were highly viable, with enhanced cytoskeletal extensions (El-Fiqi et al. 2013). Hutmacher and co-workers (2010) used totally different approach to outweigh shrinkage problem and seeded cells in a synthetic PEG-based platform and compared it with a hydrogel made of rat-COL I. Both hydrogels show colony formation over the first week of culture, but the biomimetic PEG gel did not lose size and shape whereas the collagen gel showed significant shrinkage in the long-term culture. Further pros of PEG hydrogel are design flexibility of biochemical and biological characteristics combined with the high fabrication reproducibility which enables the influence of incorporated biomolecules and/or protease substrates on the behavior of cells cultured in

3D to be studied and might make them superior to collagen and other natural hydrogels (Hutmacher 2010).

As a summary it was seen in quite early stage that hydrogels used were not optimal for vibration loading tests since shrinkage of the gels made them float freely and unrestrained in a culture medium. Therefore, it was difficult to estimate effects of the vibration loading to the cell behavior and proliferation alone. In future it would be advisable to use mechanically improved collagen matrixes or totally different biomaterials with better properties. Additionally, lower cell seeding densities could be tested thus cells do not colonize gels so fast.

6.3 Vibration loading increased the proliferation of adipose stem cells in 3D culture

Findings published previously by Tirkkonen at al. (2011) suggest that the stimulatory effect of vibration loading in 2D osteogenic culture conditions promotes differentiation of hASCs towards bone-forming cells. There was a notice, that vibration did not affect cell number or viability. However, results gained from this study indicate that hASC's proliferation rate is different in static and vibrated 3D cultures. Furthermore, the mechanical stimulation was found to have a more dominant role on hASC proliferation, with vibrated cultures promoting greater hASC proliferation with all cell lines at first time point. When gels started to shrink exceedingly in experiments I and II this impact was lost at second time point. However, the increase in cell count was still seen in experiment III with cells having a slower proliferation rate. Increase in the proliferation rate as a response to the vibration loading was also seen by other groups in their studies with BMSCs and ASCs in 2D environments (Kim et al. 2012, Uzer et al. 2013).

Since Tirkkonen and co-workers used similar vibration parameters and osteoinductive condition in their studies it may be infer that 3D culture conditions used in this study had some effect on increased proliferation rates when cells were exposed to HMHF vibration. More tissue like 3D hydrogels were used to avoid shear stress cells experience in 2D cultures or with hard materials. Interestingly Uzer and co-workers (2013) observed greatest increases in cell number for the lowest fluid shear measured. As noted previously vibrated gels with higher proliferation rate shrank first since gels could not resist growing cell population. Notable, contraction of the gels also made them denser. Cheema and Brown (2013) found in their study that cell proliferation rates were measurably higher in dense collagen gels.

6.4 Effects of vibration loading on osteogenic behavior of adipose stem cells in 3D collagen hydrogels were cell line dependent

Osteogenic capacity of the cells was first visualized with Osteoimage Mineralization assay. Similar calcification in both vibrated and static conditions was observed in the experiment III, but cells in experiment I showed no calcification in static conditions. There can be variations between cell response to the differentiation conditions and vibration in different cell lines. This is more closely discussed in section 6.7. With experiment III no notable difference between vibrated and static cultures in the amount of hydroxyapatite was observed visually. In future, it would be advantageous to quantify the mineralization levels to see if there is any disparity.

Early osteogenic marker ALP activity was elevated in experiment I and III samples at first time point, but when normalized with cell number the elevated ALP activity was noticed to be probably due to increased proliferation. Increased levels of ALP activity were observed only in the experiment III at first time point when compared to BM control. However this result arose from OM medium used since no difference between vibrated and static OM cultures was seen. Thus, vibration did not change the activity of osteoblast-specific marker ALP and expression levels of ALP gene in the Quantitative real-time PCR samples supported this observation. Further, vibration had no effect on the other osteogenic markers genes Runx2A and COLL1, but rather suppressed the mRNA expression compared to static OM cultures. Only experiment III cells showed slightly increased Runx2A levels in the first time point compared to static OM culture. On the other hand, osteogenic marker gene Dlx5 expression was elevated in all three experiments in the vibrated cells. None of the earlier vibration studies have measured the expression of Dlx5 with vibrated cells, but other osteogenic marker gene expression levels have earlier shown to be frequency dependent (Zhang et al. 2016). It might be that genes investigated were not active anymore or were not activated yet when evaluated. Hence, vibration parameters used should be examined more carefully (see section 6.6).

As Holleville et al. (2007) showed Dlx5 is a specific marker of osteogenesis. Most of the early and late markers of osteoblast differentiation could be a direct target of Dlx5 secreted by cells in early state of osteogenesis as well as later on in mineralization phase (Holleville et al. 2007). On the other hand Dlx5 has also shown to appear before osteogenic differentiation (Lee et al., 2003). In the skull, Dlx5 is expressed in neural crest-derived osteoblasts progenitors, before the onset of skull osteogenesis (Holleville et al., 2003). But in addition to this early e.g. before initiation role, the basal levels of Dlx5 expression observed

by Ryoo et al. (1997) in fetal osteoblast cell lines were further upregulated during the late mineralization stage. Since mineralization has already occurred within cells in second time point it is considered that elevated levels of Dlx5 observed in this study with all vibrated cell lines are proof from late, rather than early osteogenic differentiation. Thus it might be that lower ALP, Runx2A and collagen 1 levels are related to the fact that differentiation is faster with vibrated cells and expression of these genes is already decreased. However high degree of mineralization and higher levels of osteogenic marker gene Dlx5 expression are related to osteogenesis, so that can be concluded that vibration effects on osteogenesis at least by elevating Dlx5 expression. Mineralization in experiment III cultures was the result of the media used rather than due to vibration since it happened in both conditions. Nevertheless, enhanced mineralization observed with vibrated experiment I cells could indicate that some cell lines benefit more from stimulus received outside than other.

6.5 Adipogenesis was not inhibited by vibration loading

Analysis of the expression of adipogenic marker gene aP2 revealed that in the presence of OM and vibration stimulus, two out of three hASC lines studied expressed remarkably high levels of this marker gene. As in both time points with all cell lines vibration induced elevated expression of osteogenic marker Dlx5 and, at second time point, remarkably high levels of aP2 with cell lines HFSC 2/15 and HFSC 11/15. Expression levels of both osteogenic and adipogenic markers varied among hASC lines.

Although the studies discussed earlier have showed a reduction in adipogenic gene expression in MSCs, inhibition in the Adipogenesis was not seen in this study since adipogenic marker aP2 was also elevated in vibrated cells. This kind of effect was not seen by Tirkkonen et al. (2011) in their study. Since cells were seeded inside the hydrogels the experiment platform in this study differ from the vibration experiment performed on 2D culture plastics. Similar observation, between 2D and 3D conditions were done by Jung and al. (Jung et al. 2016) in their study with different hMSC-seeded hydrogel composites. They found that the most outstanding difference in hMSC differentiation between 2D and 3D was in adipogenic differentiation. All studied 3D environments, especially one with collagen I upregulated adipogenic PPARG gene expression and led to the query of the expression of PPAR-γ and FABP4. Also oil red O staining performed revealed fat droplet formation. Furthermore in the study conducted by Oh and co-workers (2011) effect of LMHF horizontal vibration on 3T3-L1 pre-adipocytes was investigated. They concluded that 20 and 30 Hz vibration decreased cell proliferation and increased adipogenic gene expression in the cells

already of the adipose lineage. Also Vanhatupa et al. (2015) found that the hASC donor lines differentiating towards bone in response to BMP-2 showed increased expression of the adipogenic marker gene aP2, suggesting that all the hASCs, probably related to their tissue of origin, possess some adipogenic aptitude. Kang and co-workers demonstrated that the osteogenesis and adipogenesis of C3H10T1/2 cells upon BMP-2, BMP-4, BMP-6, BMP-7 and BMP-9 stimulation was mutually exclusive even though the cells undergoing osteogenic and adipogenic differentiation were closely located (Kang et al., 2009). This might suggest that also in this study aP2 expressing subpopulation of the hASCs was distinct from the subpopulation committing towards osteogenesis in the same sample.

These results suggested that vibration could induce osteogenic as well as adipogenic differentiation in hASCs. Furthermore, some hASC lines clearly had a greater tendency toward both adipogenic and osteogenic differentiation. This indicates that some cell lines possess good differentiation capacity despite the stimulus given. Likewise, Vanhatupa et al. (2015) observed heterogeneity in the differentiation capacity within different donor cell lines, with simultaneous mineralization and lipid formation seen in some phase of the differentiation process. In addition to external signals, cell shape can also influence the process of adipogenesis (Lowe et al. 2011). Since aP2 is a late marker of adipogenesis could it be that cells inside the hydrogel direct toward adipogenesis and cells on and under the gels toward osteogenesis. Whereas rounded MSCs are more likely to become adipocytes, widely spread cells favors osteogenesis (Feng et al., 2010; Kilian et al., 2010). Furthermore, a limitation in space for the cells to proliferate has been suggested to be beneficial for the adipogenesis over osteogenesis of preadipocytes (Cristancho and Lazar 2011). It may be that gel shrinkage also promotes Adipogenesis.

Lee et al. (2013a) studied relationships between osteogenic and adipogenic differentiation of bone marrow mesenchymal stem cells and examined the regulatory role of Dlx5 in adipogenic differentiation. They observed that adipogenic stimuli suppressed the expression levels of Dlx5 in human bone marrow MSCs. Furthermore Dlx5, when over-expressed suppressed adipogenesis by decreasing the transcriptional activity of CREB and C/EBPα in a dose-dependent manner (Lee et al. 2013a). They stated that Dlx5 plays an important regulatory role in fate determination of bone marrow MSCs toward the osteoblast lineage through the direct stimulation of osteoblast differentiation and inhibition of adipocyte differentiation (Lee et al. 2013a). According to above-mentioned and to Baek (2013) in bone marrow Dlx5 enhances osteoblastogenesis through upregulation of the expression of Runx2 and osteoblast marker genes while suppressing adipogenesis through the downregulation of

PPARγ expression. These studies suggest that Dlx5 exerts anti-adipogenic activity and plays an important regulatory role in fate determination of MSCs through the stimulation of osteoblastogenesis. However, in this study adipogenesis was not inhibited but increased even if Dlx5 was expressed.

These results may imply that there might be two populations of cells in hydrogels and other goes through adipogenesis while others differentiate towards osteogenic lineage. Owing to cell origin adipogenesis may occur coincide to the osteogenesis. Moreover, it is also possible that the vibration stimulation is not specific enough stimulant of osteogenic differentiation or that the 3D setting suppresses the stimulus.

6.6 Effective parameters of vibration loading

Since cells behave differently in vibrated cultures than in static and effects of vibration were quite similar with all cell lines it can be considered that hydrogels were able to transfer vibration loading to the cells. Question is what kind of vibration parameters should be used to get the best outcome? As well as too exiguous also too excessive mechanical stimulation can disturb the cells mechanobiological signaling pathways and cause damage in tissue structure.

Only a limited number of studies have reported the effect of vibration loading specifically on hASCs (Tirkkonen et al. 2011, Pre et al. 2011, Park et al. 2012, Uzer et al. 2013) and none of them in 3D model. Because of that and the varying vibration configuration used in previous studies, the parameter values chosen for this research were experimental. Compared to most studies conducted in 2D both acceleration and frequency used in this study, as well as the number and the duration of the stimulation periods were significantly higher. These exaggerated forces were applied since signal transfer in 3D cell cultures was considered inferior to signal transfer inside the bone lacunae. Study conducted by Tirkkonen et al. (2011) also demonstrated that hASC benefitted from forces applied and did not suffer under HMHF conditions. However, the joint effect of growing cell population and mechanical stress resulted in hydrogel detaching. Thus, it might be advisable to cut down at least vibration periods used. Actually all *in vivo* and *in vitro* studies summarized used only one or two effective periods per day or significantly lower durations than 30 minutes and nevertheless perceived enhanced osteogenesis. Also various frequency and acceleration combinations have shown enhanced osteogenesis and study setting optimizing would require further comparison to find most effective parameters that support differentiation, but are still bearable for the cells and biomaterial. There is some evidence that accelerations higher than 1.0g in WBV applications may cause some negative effects on bone (Kiel et al. 2015, Muir et

al. 2013). Hence, should accelerations higher than this also avoid with cell studies to ensure optimal outcome without unwanted side effects. On the other hand, the idea of this study was to exaggerate the forces used to see the direct effect of the mechanical loading on the cells. After all, cell cultures lack the signal amplifying mechanisms like lacunae in the bone.

However, parameters used should support osteogenesis and enable constant vibration sensed by cells embedded in the biomaterial. Since hydrogels in this study shrank and came loose at different times the effect of vibration parameters on osteogenesis was challenging to evaluate. It was not evident that only forces influencing the cells were the defined.

6.7 Donor variation effects greatly on hASC differentiation and clinical use

As seen in WBV studies different study groups react differently on vibration. Previous studies in healthy children (Harrison et al. 2015) and in young women with osteoporosis (Gilsanz et al. 2006) demonstrated that low intensity vibration was anabolic to the skeleton. Additionally, in a study of recently post-menopausal women (Rubin et al. 2004) there was less bone loss in the active group of women with high adherence and low body weight than the control group. In contrast, there is evidence that healthy postmenopausal women ages 44 - 79 years randomized to 12 months of WBV showed no effects on changes in BMD when compared to a group who received no stimulation (Slatkovska et al. 2011). Furthermore, randomized trials of daily WBV in older adults using low magnitude (Kiel et al. 2015) or high magnitude (1.6 g to 2.2 g) vibration (Verschueren et al. 2011) did not demonstrate evidence of significant beneficial effects on bone mineralization density.

The problem of donor variation has also been noticed in multiple *in vitro* studies and therefore reduced differentiation capacity of ASCs could compromise their autologous use in TE applications. There may be reasons why cell lines behave differently and beneficial effects on the osteogenesis vary between cell lines. While effects of the WBV differed by age, BMI or diseases like diabetes and metabolic disorder or hormonal status of the donor—similarly the donor variation of hASCs may stem from different variables. Harvesting procedure does not have effect on the yield or characteristic of ASCs, but there is depot-dependent and donor-dependent variability in the adipogenic and osteogenic differentiation capacities of the ASC populations derived from different sources (De Girolamo et al. 2013, Russo et al. 2014). For example, the differences in body fat distribution, the obesity-related co-morbidities and BMI affect ASC's proliferation and differentiation potential (De Girolamo et al. 2013, Frazier et al. 2013, Oliva-Olivera et al. 2015). Additionally, cells obtained from the older donors display senescence associated features which all impacts stem cell function and are associated with

significantly reduced viability, proliferation and differentiation potential in aged ASCs compared to young ASCs (Choudhery et al. 2014, Kornicka et al. 2015). Furthermore, the regenerative potential of MSCs from diabetic (Cramer et al. 2010) and osteoporotic (Jiang et al. 2014) donors is known to be reduced.

Overall, studies with ASCs suggest that donor variation and depot selection are important factors to consider when applying ASCs in tissue-specific cell-based regenerative therapies. As it is proven, cells from different donors behave differently and thus signals induced, like vibration, generate divergent response in receiving cell population.

7 CONCLUSIONS AND FUTURE PROSPECTS

In this study the 3D environment and mechanical methods, i.e. HMHF vibration loading for the osteogenic differentiation of hASCs were evaluated. On the basis of this study the main findings and conclusions are:

Ia. Selection between three different hydrogels was done in phase II. Hyaluronic acid based hydrogels alone or combined with collagen I were not suitable for further studies. Both, cell viability and consistency of the gels were compromised in these materials. According to the tests with collagen I extracted from rat tail and bovine origins both hydrogels supported the viability, attachment, and growth of hASCs cultured in basal medium. Rat tail collagen I was selected for the vibration experiment due to lesser contraction observed.

Ib. hASCs from all donor lines, in all culture conditions stayed viable in rat-COL I when fabrication parameters, especially pH were checked properly. Although it was seen in quite early stage of vibration experiment that mechanical properties of collagen I used were not optimal for vibration loading tests since shrinkage of the gels made it difficult to evaluate effects of the vibration to the cells. The greatest challenge in the future 3D studies under vibration loading will be to find suitable hydrogel. Selected hydrogel should resist pulling forces produced by increasing cell density and mechanical loading generated by vibration and still support cell viability and osteogenesis. Reliable results are only gained with hydrogels that do not shrink and detach from the well walls. In future, it would be wise to use mechanically improved collagen matrixes or totally different biomaterials with more suitable properties.

II. Vibration did not seem to have an impact on cell viability or morphology compared to static control, but it did increase notable cell proliferation in all cell lines. Since cells behave differently in vibrated cultures than in static and effects of vibration were quite similar with all cell lines it can be considered that hydrogels were able to transfer vibration loading to the cells. Results obtained from this study indicate that hASC's proliferation rate differs in static and vibrated 3D cultures. Furthermore, the mechanical stimulation was found to have a more dominant role on hASC proliferation at first the time point. As gels started to shrink exceedingly this impact was lost at the second time point with experiment I and II while increase in cell count was still seen with more slowly proliferating experiment III cells. In future shrinkage of the collagen gels could be delayed by using lower cell seeding densities or less aggressive vibration.

The effect of vibration stimulation on hASC differentiation in the selected 3D hydrogel remains unclear. At both time points with all cell lines vibration induced elevated expression of osteogenic marker Dlx5 and at second time point, remarkably high levels of aP2 with cell lines HFSC 2/15 and HFSC 11/15. Expression levels of both osteogenic and adipogenic markers varied among hASC lines. Nevertheless, vibration loading stimulated differentiation of hASCs towards bone-forming cells though differentiation was not that specific. It is possible, that variable stiffness of the 3D environment disturbed cell signaling or suppressed stimulation. Additionally, vibration parameters can have significant effect on 3D matrix and hASC behavior, which should be taken into account when designing further studies to optimize culture conditions.

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